PHYCOLOGICAL STUDIES

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R. MALCOLM BROWN, JR. AND HAROLD C. BOLD

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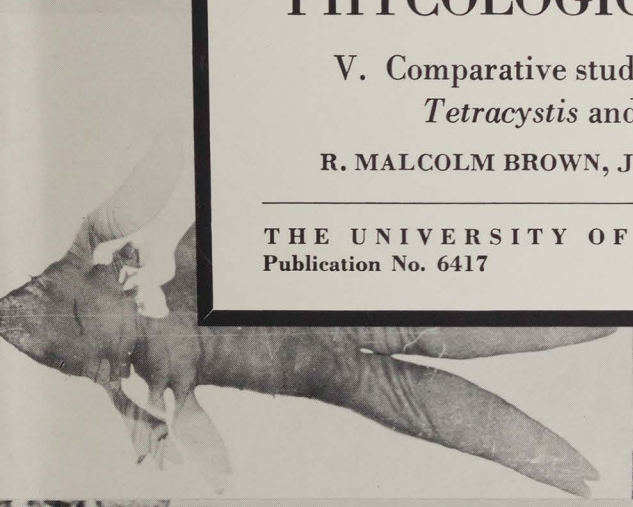

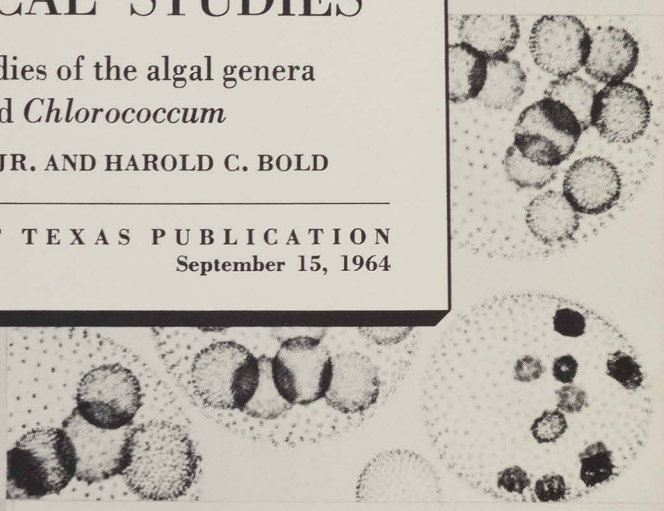

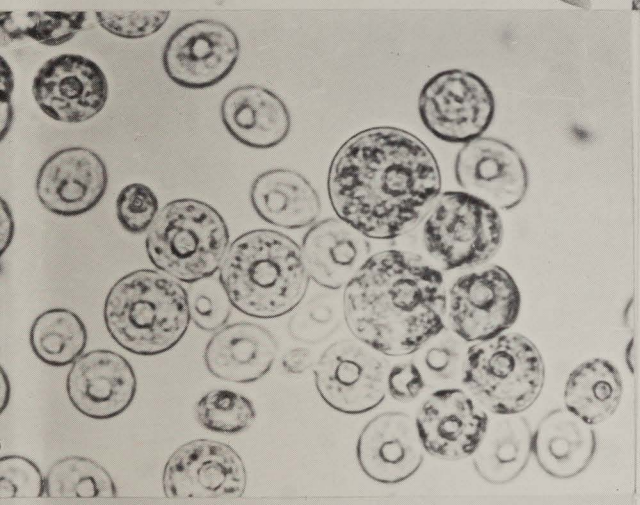

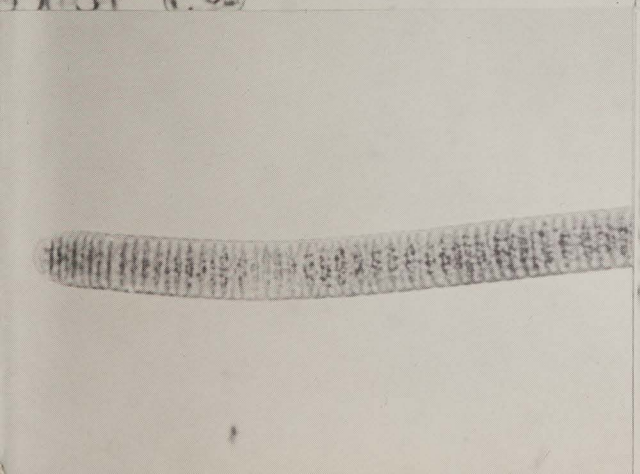

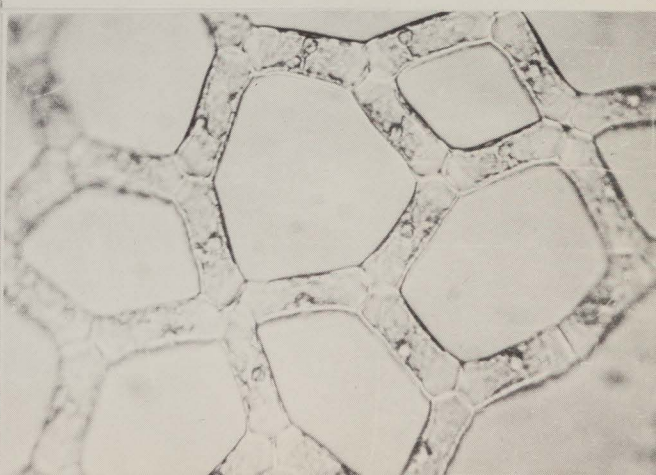

Phycological Studies

V. Comparative studies of the algal genera *Tetracystis* and *Chlorococcum*

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(Immunological studies with the collaboration of Richard N. Lester[2])

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I. General Introduction

During the past 5 years, the senior author has been devoting attention to air-borne microorganisms, especially algae, with reference to their abundance and heterogeneity. Since January 1, 1963, he has been sampling the air daily on the the campus of The University of Texas and has been isolating the algae from these samples into axenic culture in view of their probable allergenic properties (McElhenny, Bold, Brown, and McGovern, 1963). In addition to the Austin samples, algae have been cultured from air samples taken at various locations in the continental United States (21 states), Hawaii,[1] and Mexico.[2] These procedures have revealed a hitherto unexpectedly rich and diversified algal component of the atmosphere (Brown, Larson, and Bold, 1964) and have brought into clear focus the desperate need for taxonomic and monographic studies of soil and air-borne algae *per se* and as a prelude to investigations of the allergenic properties of these organisms.

During the course of these investigations, a number of cultures were assembled of a unicellular, chlorophycean alga, frequently present in the air, the alga being characterized by undergoing vegetative cell division (*sensu* Herndon, 1958) and clearly unlike any alga previously described. These isolates have been included in the newly proposed genus *Tetracystis* (Chlorosphaeraceae, Chlorosphaerales) to be described in this paper. In addition to the cultures of *Tetracystis* isolated from air, a number have been isolated from soil samples, soil itself clearly being the source of most air-borne algae.

From approximately 50 isolates of *Tetracystis,* 10 cultures representing 9 species, have been chosen for intensive study and characterization. In addition, the writers have uncovered evidence which indicates that 3 other algae described earlier as species of the genus *Chlorococcum* (Chlorococcaceae, Chlorococcales) are probably also members of the chlorosphaeralean genus *Tetracystis.* According, 13 organisms have been studied intensively and on the basis of these studies, 9 new species[3] and 3 new combinations have been proposed.

It has become increasingly clear through our expanding knowledge of the soil algal flora (Trainor and Bold, 1953; Starr, 1955; Herndon, 1958b; Arce and Bold, 1958; Deason, 1959; Deason and Bold, 1960; Chantanachat and Bold, 1962; Mattox and Bold, 1962; and Bischoff and Bold, 1963) that there are numerous taxa of nonmotile, spherical unicellular algae with biflagellate, motile stages. That these organisms cannot be identified by direct inspection in mixed collections and

[1] Courtesy of Dr. and Mrs. Wilson S. Stone, Department of Zoology, The University of Texas, Austin, Texas.

[2] Courtesy of Dr. C. J. Alexopoulos, Department of Botany, The University of Texas, Austin, Texas.

[3] Including two isolates of *T. aeria.*

cultures is conceded by all who have examined such mixtures. Accordingly, the methods of study are of necessity microbiological, with attention to morphological and, increasingly, to physiological attributes of the organisms in axenic culture.

This methodology has been applied to the algae which form the subject of this report. It has been augmented by including electron-microscopic and immunochemical methods. It is the writers' opinion that these enriched and expanded procedures have provided considerable insight into the taxonomy of the *Tetracystis* species under consideration, while at the same time contributing data of broader biological significance and interest. These data will be discussed under 3 major headings, namely: (1) Morphology, taxonomy, and physiology of *Tetracystis* gen. nov.; (2) Electron microscopy of *Tetracystis* and certain *Chlorococcum* species; and (3) Immunochemical studies of *Tetracystis* and *Chlorococcum*. Presentation of the data under each of these headings will be preceded by an account of the materials and methods employed.

II. Morphology, Taxonomy, and Physiology of Tetracystis Gen. Nov.

A. MATERIALS AND METHODS

Four of the 13 intensively studied isolates of *Tetracystis* were collected from the air by exposing Petri dishes (100 × 15 mm) of solidified (1.6%) "Bold's Basal Medium" (BBM) (Bischoff and Bold, 1963) at various locations for given periods. The remaining 9 isolates from the soil were either provided to the writers in unialgal or axenic condition, or isolated from soil in the following manner: 5 g of a given soil sample were inoculated into a 125-ml Erlenmeyer flask containing 50 ml of sterile liquid BBM.

"Bold's Basal Medium" (BBM) was prepared as follows: macroelements were supplied in the form of 6 stock solutions by dissolving the indicated weight of the following salts into 400 ml distilled or de-ionized water:

$NaNO_3$	10.0 g
KH_2PO_4	7.0 g
K_2HPO_4	3.0 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$CaCl_2 \cdot 2H_2O$	1.0 g
NaCl	1.0 g

Ten ml of each stock were employed for each liter of final solution.

Minor (trace) elements were supplied in the form of the 4 following stocks:

EDTA Stock Solution

50 g EDTA (Ethylenediaminetetraacetic Acid) and 31 g KOH were diluted to 1 liter with de-ionized or glass-distilled water.

H-Fe Stock Solution

4.98 g $FeSO_4 \cdot 7H_2O$ were diluted to 1 liter with acidified water. Acidified water was made by adding 1 ml concentrated H_2SO_4 to 999 ml de-ionized or glass-distilled water.

H-Boron Stock Solution

11.42 g H_3BO_3 were diluted to 1 liter with de-ionized or glass-distilled water.

H-H_5 Stock Solution

8.82 g $ZnSo_4 \cdot 7H_2O$
1.44 g $MnCl_2 \cdot 4H_2O$
0.71 g MoO_3
1.57 g $CuSO_4 \cdot 5H_2O$
0.49 g $Co(NO_3)_2 \cdot 6H_2O$ were diluted to 1 liter with acidified water (as above).

One ml of each stock solution was added to a liter of the final solution.

Following exposure to the air for various lengths of time, the Petri dishes with BBM were incubated from 2 to 4 weeks under standard conditions of culture.[1] Likewise, the Erlenmeyer flasks containing soil were incubated from 2 to 6 weeks, after which a portion of the supernatant was streaked across 100 × 15 mm Petri dishes of solidified BBM. After 2 weeks' incubation under standard conditions of culture, macroscopically visible colonies were identified and isolated into unialgal culture on agar slants of BBM.

Other media used for growing fastidious organisms included soil extract agar and proteose agar (Starr, 1964).

The morphology and life-cycle phases of *Tetracystis* were studied by making fresh mounts and hanging-drop preparations from cultures which were growing on agar slants or Petri dishes. Agar slant cultures of *Tetracystis* were maintained as stocks, and when cultures were desired for study, they were transferred into fresh agar slants of BBM. Cultures prepared for study on Petri dishes were inoculated from actively growing agar-slant cultures. Five ml of sterile, liquid BBM were aseptically added to each agar slant culture and the algal material gently removed from the agar surface into the liquid phase by means of a sterile platinum loop. The cells were further dispersed into a homogeneous suspension by a 5–10 sec treatment in an ultrasonic water bath.[2] Six drops of this homogeneous suspension were transferred aseptically from the tube to the surface of agar in Petri dishes by a disposable Pasteur pipette. The inoculum was then vigorously swirled to disperse the algae evenly, and the Petri dishes were inverted and maintained under standard conditions for from 2 to 4 weeks. If the $NaNO_3$ concentration was increased to 3 times that of BBM, the Petri dish cultures could be maintained in log phase of growth up to 3 weeks, as compared to 10–14 days on the standard basal medium.

[1] Standard conditions of culture: illumination of 250–300 ft-c intensity; a 12–12 hr diurnal, light-dark cycle; and a temperature range of 19–22° C.

[2] Di-sontegrator, System 80; Model G–80C1; Ultrasonic Industries, Inc., Albertson, New York.

Tetrad formation occurred in greatest frequency in cultures exposed for about 6 hr to light of the 12–12 diurnal dark-light period. Zoospore formation usually could be evoked in actively growing cultures by placing a culture (growing on freshly poured agar) in an uninterrupted dark period of 5–8 hr, followed by a continuous light period of 1–3 hr. However, some species of *Tetracystis, T. texensis* for example, were very fastidious. Here, zoospore formation could be effected only by repeated transfer from solidified BBM to liquid BBM and by manipulating the day-length cycle.

Cell-wall thickness and the presence or absence of gelatinous matrices were determined with India ink and/or a weak aqueous solution of Methylene blue. Various concentrations of I_2KI were used to determine the presence of starch, the position of the nucleus, and the number and length of zoospore flagella.

Limited cytological studies were made by fixing the algae (which had been attached to a microscope slide by egg albumin) in a freshly prepared solution of 1 part glacial acetic acid to 3 parts absolute ethanol for 30 min. Then the slides were flooded with acetocarmine prepared according to the method of Cave and Pocock (1956) and passed over a low flame until vapors arose from the stain. After the heating process, the slides were drained and observations were made immediately. No permanent preparations were made.

Various physiological and certain morphological tests require the use of axenic cultures. These were achieved by processing unialgal cultures of *Tetracystis* through certain physical and chemical treatments which are described in detail by Brown and Bischoff (1962).

A number of media were employed repeatedly for purity of cultures and for studying comparative growth. These included Proteose Agar (Starr, 1964); Difco Nutrient Agar; Difco Nutrient Broth; Difco Thioglycollate Broth, and Yeast Extract Agar. The latter was prepared by adding 5 g of yeast extract powder to 1 liter of distilled or deionized water and 16 g of agar.

Other media were employed specifically for comparative study of certain physiological attributes. Their composition and preparation are described at appropriate sites in the body of the paper.

Observations of colony characteristics and isolation of single cells or colonies for axenic cultures were made with a Bausch and Lomb stereroscopic binocular microscope. Photomicrographs were taken with a 35-mm Zeiss-Winkel camera attached to a Bausch and Lomb microscope with apochromatic objectives. Macroscopic pictures were made with a Zeiss Super Contaflex 35-mm single-lens reflex camera.

B. GENERIC CHARACTERIZATION

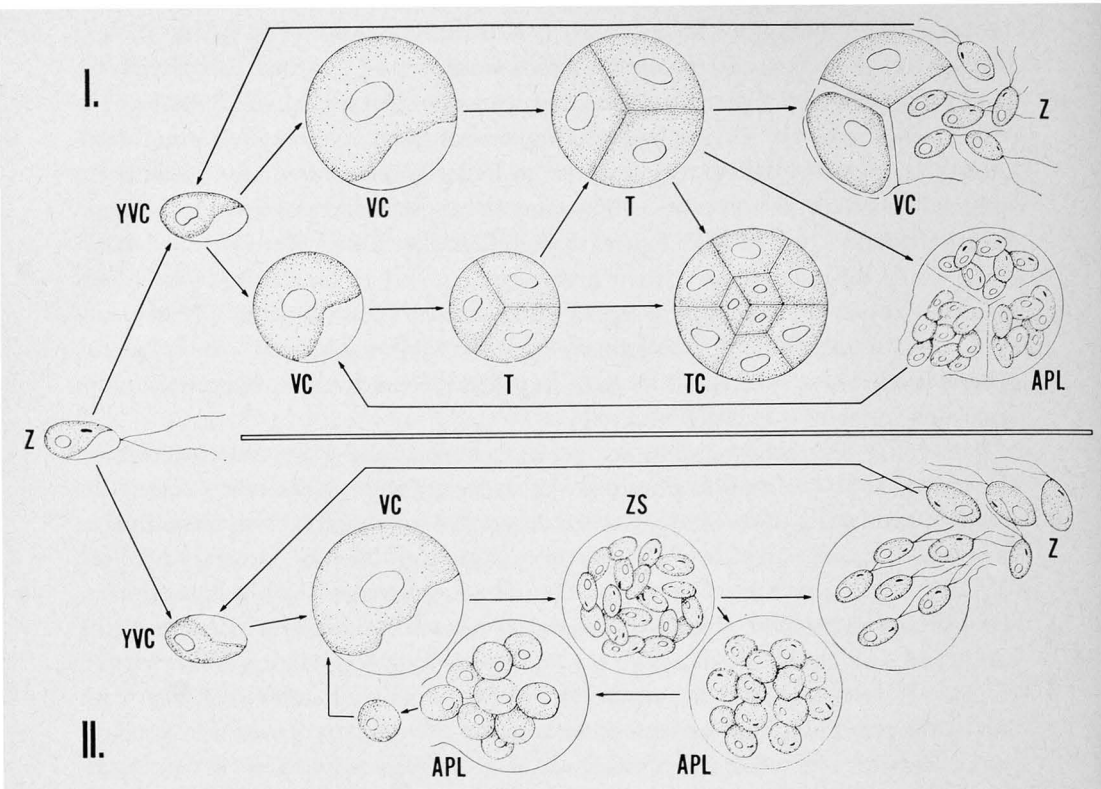
The fact that many of the 13 algae under consideration at some stage in their life cycle occur as tetrahedral tetrads of vegetative cells (Text-fig. 1) suggested at once to the writers that they were species of the little known and infrequently encountered genus *Borodinella* (Miller, 1927). However, continuing study of the

isolates, careful reading of Miller's paper, and examination of his figures made it certain that the algae under consideration could not be assigned to *Borodinella.* Miller described and figured for the latter an axile chloroplast, while those of the writers' organisms are always parietal, however massive. Although it is not absolutely certain from Miller's paper (and no living type cultures are available for verification), that the zoospores of *Borodinella* become spherical upon quiescence, there is evidence from Miller's figures that, in fact, they did so. *Borodinella,* accordingly, would differ from the writer's organisms, not only in the nature of its chloroplast, but also in the fate of its zoospores, 2 characters shown by Starr (1955), and a number of subsequent investigators, to be reliable taxonomic criteria at the generic level. These considerations have impelled the assignment of the algae under discussion to the new genus *Tetracystis.*

Tetracystis, like *Chlorosarcinopsis,* clearly belongs to the order Chlorosphaerales (Herndon, 1958). Herndon proposed this order for those unicellular, nonmotile, zoospore-producing Chlorophyceae with vegetative cell division. This last attribute has been discussed critically by Herndon (1958) and later by Deason and Bold (1960). As conceived by Herndon, vegetative cell division involves partitioning of a cell and deposition of new wall material in such fashion that portions of the parent cell wall clothe the daughter protoplasts, at least immediately following cell division. Deason and Bold believed that contiguity of the daughter cell wall with that of the parent was not necessarily essential to the concept of vegetative cell division. Instead, vegetative cell division was conceived by them to involve intervening cell-wall deposition between each mitosis and cytokinesis. In contrast with cells dividing non-vegetatively (to form zoospores and aplanospores), rapidly occurring nuclear divisions and cytokineses are followed by cell wall deposition only after all nuclear and protoplast divisions have been completed, according to Deason and Bold.

The writers have found that both Herndon's and Deason and Bold's concepts of vegetative cell division would not include unequivocally all the species of *Tetracystis,*[1] yet this genus clearly seemed to belong in the Chlorosphaerales because of the presence of tetrads of daughter vegetative cells (Fig. 43) and complexes of the same (Fig. 22). For this reason, the writers sought more evidence which would aid in the stabilization of taxonomic characteristics for the order Chlorosphaerales and found in *Tetracystis,* along with all presently known chlorosphaeralean algae, an additional precise, ordinal attribute for the Chlorosphaerales, namely, the following: intercalated between the motile and vegetative phases in a given cycle (Text-fig. 1), members of the Chlorosphaerales have a nonmotile phase, in which the division products of vegetative cells are neither motile nor potentially motile like aplanospores. These products, daughter vegetative cells, may become dissoci-

[1] Some species of *Tetracystis* lose parent and daughter wall contiguity immediately after wall formation; some species form tetrads of daughter cells directly, without intervening diad formation.



Text-fig. 1

Diagrammatic representation of the life cycles of the Chlorosphaerales, exemplified by *Tetracystis* (I and II) and the Chlorococcales, exemplified by *Chlorococcum* (II only). Z = zoospore, YVC = young vegetative cell, VC = vegetative cell, ZS = zoosporangium, APL = aplanospore, T = tetrads of daughter vegetative cells, both small and large, and TC = tetrad complexing. The vegetative cells of *Chlorococcum* have the capacity to form only zoosporangia which, in turn, produce zoospores or aplanospores (II). The vegetative cells of *Tetracystis* also may form zoospores or aplanospores (II), and, in addition, they may form tetrads of nonmotile, daughter vegetative cells (1) which do not have the capacity for potential motility. These tetrads may be of 2 sizes at maturity. The daughter vegetative cells within the tetrad may form zoosporangia which, in turn, form zoospores or aplanospores, or they may continue to produce tetrads of nonmotile daughter cells resulting in tetrad complexes (TC). The tetrad complexes may form zoospores or aplanospores (not shown). Daughter vegetative cells may be released from tetrads or tetrad complexes, either very soon after their formation, or only very late, depending on the species.

ated at the diad, tetrad, or octad levels, or they may remain in association to form complexes. The products of the nonmotile phase are similar to autospores[1] in that both are nonmotile, and unlike aplanospores in that the daughter vegetative cells in chlorosphaeralean tetrads have not developed from zoosporic antecedent.

[1] Until autospore formation has been more thoroughly studied electron microscopically than by Murakami, Morimura, and Takamiya (1963), no final decision can be made regarding whether the vegetative cells are identical with autospores in their ontogeny. The writers distinguish aplanospores and autospores on the basis that aplanospores arise (in ontogeny) from zoospores or potential zoospores, which seemingly is not true of autospores.

By contrast, it will be recalled that nonmotile vegetative cells of chlorococcacean algae always reproduce by forming either motile (zoospores or gametes, or both) cells or nonmotile, but potentially motile, cells (aplanospores) (Text-fig. 1).

Tetracystis gen. nov.

Cellulae vegetativae seiunctae aut binae quaternae, octonae, senae denae, etc., cellulis filiabus aggregationum primum associatis, interdum deinde dissociantibus. Cellulae chloroplastrum cavum plus minusve solidum, parietalem, per fissuras saepe transversum, pyrenoideo praeditum, habentes.

Reproductio per divisionem in duas-octo cellulas vegetativas, sine potestate mobilitatis directae, eo modo differans ab aplanosporis quas cellulae quoque efficere possunt. Reproductio asexualis per zoosporas per cellulas omnis aetatis (cellulis filibus vegetativis tetradis inclusis), formatas, zoosporis admodum quiescentibus sphericis non factis.

Reproductio sexualis, cum apparet, per gametas biflagellatas.

Vegetative cells isolated or in groups of 2, 4, 8, or in multiples of 2 or 4, the daughter cells of the groups associated at first, sometimes secondarily dissociating. Cells with a hollow, more or less massive, parietal chloroplast, often transversed by fissures; with a pyrenoid.

Asexual reproduction by division into 2–8 vegetative cells which lack the potentiality of direct motility; thus differing from aplanospores which arise from motile or potentially motile precursors. Asexual reproduction also by zoospores formed by cells of all ages (including the daughter vegetative cells of the tetrad), the zoospores not becoming spherical immediately upon quiescence.

Sexual reproduction, when present, by biflagellate gametes.

Unlike *Chlorosarcinopsis* and *Chlorosarcina,* which may form regular aggregates of packets, *Tetracystis* is characterized by the presence of tetrads of nonmotile daughter vegetative cells (or diads and octads) which may or may not cohere to form irregular complexes (Text-fig. 1; Fig. 80). Furthermore, the zoospores of *Tetracystis* do not become spherical immediately upon quiescence (Fig. 31). Unlike *Chlorococcum, Tetracystis* forms nonmotile daughter vegetative cells (Text-fig. 1; Fig. 60) which are not aplanosporic in origin and nature.

C. SPECIFIC CHARACTERIZATION

Attributes of the newly named species of *Tetracystis* follow.

Tetracytis aeria sp. nov. (C–6)[1] (Fig. 13–18, 89–92)[2] (Type species)

Cellulae vegetativae iuvenes ellipsoideae ad ovatus, in cellulae vegetativas sphericas 14–16 μ diam. celeriter maturescentes. Membrana cellulae aliquantulum incrassata (0.5–1.0 μ in culturis duarum hebdomadum aetate), et magis incrassata (2–4 μ) in

[1] Designation of isolates in authors' culture collection.

[2] Figures illustrating ultrastructure are not cited here.

culturis trium mensium aetate. Chloroplastus solidus, aliquot fissuras radiantes non profundas praebens, unicum pyrenoideum sphericum, 4 μ diam., multis amyli micis circumdatum, habens. Cellulae flavovirides in incrementi periodo immobili (tribus mensibus) factae. Vacuolae contractiles in cellulis vegetativis uninucleatis infrequenter observatae.

Reproductio asexualis per cellulae divisionem diades aut plerumque tetrades cellularum vegetativarum directe efficientem, quarum maturarum membranae cellulae parentis membranae conferte associatae. Complexus unius ad aliquot tetrades frequentes. Reproductio asexualis etiam per zoosporas aplanosporas. Zoosporae dolioformes (late cylindricae) 10–12 × 5 μ, nucleum anteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum pyrenoideum equatoriale stigma anterius magnum duo flagella longitudine aequa, 1–1/4—1–1/2 longi longiora quam longitudo corporis habentes.

Reproductio sexualis non observata.

Culturae duarum hebdomadum aetate in medio "agar" basali modice dilute virides; superficies coloniae levis ad aliquantulum asperam (minute granulosam) magnificatione sex et 12 plo; coloniae magnificatione nulla opaco-nitidae ad nitidas.

Origo: ex aere super locum Pampa, Texas, dictum, b. Jun. 1960.

Young vegetative cells ellipsoidal to ovoid, rapidly maturing into spherical vegetative cells 14–16 μ in diameter. Cell wall slightly thickened (0.5–1.0 μ in 2-week-old cultures) and more thickened (2–4 μ) in cultures 3 months old. Chloroplast massive, with a few shallow, radiating fissures, containing a single, spherical pyrenoid, 4 μ in diameter, surrounded by many starch grains. Cells becoming yellow-green in the stationary phase of growth (3 months). Contractile vacuoles infrequently observed in the uninucleate vegetative cells.

Asexual reproduction by cell division giving rise directly to diads or mostly to tetrads of vegetative cells, the walls of which are closely associated with the parent cell wall at maturity. Complexes of 1 to several tetrads frequent. Asexual reproduction also by zoospores and aplanospores. Zoospores barrel-shaped (broadly cylindrical), 10–12 × 5 μ, with an anterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single, equatorial pyrenoid, a large anterior stigma, and 2 flagella of equal length 1–1/4—1–1/2 times body length.

Sexual reproduction not observed.

Two-week-old cultures on basal agar medium light-green; colony surface smooth to slightly rough (minutely granular) at 6 and 12 × magnification; colonies dull-shiny to shiny macroscopically.

Source: from air over Pampa, Texas, June, 1960.

Two cultures of *Tetracystis* (C–6 and Pa–3), which are presumably the same species (*Tetracystis aeria*), have been isolated from diverse geographical localities. Isolate C–6 was made from an air collection over Pampa, Texas, 485 miles north of Austin, Texas, the source of the second isolate, Pa–3. The latter was isolated from an air collection from the campus of The University of Texas, approximately 250 ft above ground level.

It is of interest to note that these 2 isolates are virtually indistinguishable from one another morphologically and physiologically, and when compared ultrastructurally and immunochemically, no differences were detected. *Tetracystis aeria* and *T. aplanosporum* are the only 2 presently known species of *Tetracystis* in which the zoospores have anterior nuclei. *Tetracystis aeria* is very readily distinguishable morphologically from *T. aplanosporum* by: (1) smaller size of mature vegetative cells of 14–16 μ; (2) tetrad coherence to form cellular complexes; (3) cell wall thickening of 2–4 μ at 3 months on BBM agar; (4) zoospores which have a large and distinct anterior stigma; and (5) zoospores with flagella longer than the cell body length.

Tetracystis aeria reproduces asexually to form diads or tetrahedral tetrads of daughter cells (i.e., only 3 of the 4 daughter cells can be observed in a given focal plane (Fig. 122, 126). The tetrahedral tetrads are formed directly without intervening diads formation, cytokinesis not being initiated until all nuclear divisions have been completed. Diad stages are infrequently present; however, isobilateral tetrads, which originate from diads, have not been observed in *T. aeria*.

Tetracystis aggregata sp. nov. (Pc–1) (Fig. 33–40, 83–84)

Cellulae vegetativae iuvenes ellipsoideae ad ovatas, in cellulas vegetativas sphericas 15–16 μ diam. celeriter maturescentes. Incrassatio membranae cellulae in culturis duarum hebdomadum non notabilis, in culturis trium autem, mensium, aetate a 2 ad 3 μ varians. Chloroplastus solidus, fissuras radiales latas saepe praebens. Pyrenoideum 4–6 μ diam., centrale, saepe lobatum, micis amyli multis circumdatum. Cellulae in incrementi periodo immobili semper virides. Vacuolae contractiles in cellulis vegetativis uninucleatis interdum observatae.

Reproductio asexualis per cellulae divisionem et diades et tetrades cellularum vegetativarum immobilium, maturarum membranae cellulae perentis conferte associatarum, directe efficientem. Multae diades tetradesque cohaerentes, complexus magnos formantes. Reproductio asexualis etiam per zoosporas aplanosporasque. Zoosporae ovatae 4 × 10–12 μ, nucleum posteriorem duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum pyrenoideum equatoriale, stigma equatoriale ad paululo anterius et duo flagella longitudine aequa, 1–1/2 plo longiora quam longitudo corporis cellulae habentes.

Reproductio sexualis per gametas isogamicas a zoosporis morphologicaliter indistinguibiles nisi quod saepe minores. Zygotum echinatum, 15–20 μ diam., divisione quattuor cellulas vegetativas efficiens.

Culturae duarum hebdomadum aetate in medio "agar" basali atrovirides; superficies coloniae granulosa (minute botryoidea) magnificatione sex et 12 plo; coloniae magnificatione nulla siccae.

Origo: a collectione in aere ca. 83 metra alt., in campo loci Univ. Texas, Austin, dicti, m. Jul. 1960.

Young vegetative cells ellipsoidal to ovoid, rapidly maturing into spherical vegetative cells, 15–16 μ in diameter. Cell wall thickening insignificant in 2-week-old

cultures but ranging from 2 to 3 μ in cultures 3 months old. Chloroplast massive, with broad, radial fissures often present. Pyrenoid 4–6 μ in diameter, central, often lobed, surrounded by many starch grains. Cells remaining green in stationary growth phase. Contractile vacuoles occasionally observed in uninucleate vegetative cells.

Asexual reproduction by cell division giving rise directly to both diads and tetrads of nonmotile vegetative cells which are intimately associated with parent cell wall at maturity. Many diads and tetrads coherent, forming large complexes. Asexual reproduction also by zoospores and aplanospores. Zoospores ovoid, 4 × 10–12 μ, with a posterior nucleus; 2 anterior contractile vacuoles; parietal chloroplast; a single equatorial pyrenoid; equatorial-to-slightly anterior stigma; and 2 flagella of equal length, 1–1/2 times cell body length.

Sexual reproduction by isogamous gametes, indistinguishable morphologically from zoospores except often by their smaller size. Zygote echinate, 15–20 μ in diameter, undergoing division giving rise to 4 vegetative cells.

Two-week-old cultures on basal agar medium, dark green; colony surface granular (minutely botryoid) at 6 and 12 × magnifications; colonies dry macroscopically.

Source: from air collection approximately 250 ft above ground level, The University of Texas campus, Austin, July, 1960.

Tetracystis aggregata has many attributes in common with *T. isobilateralis, T. dissociata,* and *T. illinoisensis*; however, it differs significantly from *T. isobilateralis,* the species which it most clearly resembles, in: (1) the presence of a rough granular colony at 2 weeks on BBM agar; (2) strong adherence of diads and tetrads to form large cellular complexes; and (3) the presence of occasional deep, radial fissuring of the chloroplast. The latter characteristic can sometimes lead to misinterpretation of the chloroplast as axile. However, since the manifestation of deep, broad, radial fissures is not always apparent, and since the chloroplast is most typically massive, hollow, and cup-like, this organism is herein described as a species of *Tetracystis.*

Tetracystis dissociata[1] sp. nov. ("Starr") (Fig. 25–32, 87–88)

Cellulae vegetativae iuvenes ellipsoideae multae, in cellulas vegetativas sphericas ad subsphericas 14–16 μ diam. tarde maturescentes. Incrassationes internales unipolares suque ad 1 μ crass. in culturis trium mensium aetate interdum repertae. Chloroplastus nitido-viridis, parietalis, satis solidus, paucissimas fissuras non profundas praebens, unicum pyrenoideum centrale lobatum 3–4 μ diam., multis amyli micis circumdatum, habens. Culturae virides in incrementi periodo immobili (tribus mensibus) aerolis albis (cellulis defunctis) maculosae factae. Vacuolae contractiles in cellulis vegetativis uninucleatis nullae.

Reproductio asexualis per cellulae divisione raro diades, plerumque tetrades cellu-

[1] No. 128 in Culture Collection of Algae, Indiana University; isolated by Dr. Vischer and labeled "*Borodinella* sp."

larum vegetativarum, membranae cellulae parent parentis conferte associatarum, directe efficientem. Tetrades diadesve ad complexus formandos numquam associatae, sed ut diades tetradesque manentes. Reproductio asexualis etiam per zoosporas aplanosporas. Zoosporae cylindricae, 3–4 × 9–10 μ nucleum posteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum stigma equatoriale-ad-anterius, et duo flagella longitudine aequa, 1–1/2 plo longiora quam longitudo corporis cellulae habentes.

Reproductio sexualis non observata.

Culturae duarum hebdomadum aetate in medio "agar" basali nitido-virides; superficies coloniae levis homogeneaque magnificatione sex et 12 plo, colonia magnificatione nulla opaconitida.

Origo: e collectione culturarum Algarum in Univ. Indiana #128 ut *Borodinella* sp. sec. Vischer qui eam seiunxit.

Young vegetative cells ellipsoidal and numerous, maturing slowly into spherical to subspherical vegetative cells 14–16 μ in diameter. Occasional internal unipolar wall thickenings up to 1 μ thick in cultures 3 months old. Chloroplast bright-green, parietal, and rather massive, with very few shallow fissures. Chloroplast with a single, central, lobed pyrenoid, 3–4 μ in diameter, surrounded by many starch grains. Cultures green, becoming mottled with white areas (dead cells) in the stationary phase of growth (3 months). Contractile vacuoles absent in uninucleate vegetative cells.

Asexual reproduction by cell division only occasionally giving rise directly to diads and mostly to tetrads of vegetative cells which are intimately associated with the parent cell wall. Tetrads or diads never associated to form complexes but remaining at the diad and tetrad level of association. Asexual reproduction also by zoospores and aplanospores. Zoospores cylindrical, 3–4 × 9–10 μ, with a posterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single, equatorial-to-anterior stigma, and 2 flagella of equal length, 1–1/2 times cell body length.

Sexual reproduction not observed.

Two-week-old cultures on basal agar medium, bright-green; colony surface smooth and homogeneous at 6 and 12 × magnification; colony dull-shiny macroscopically.

Source: Culture Collection of Algae at Indiana University No. 128 as "*Borodinella* sp." sec. Vischer, the isolator.

This organism was obtained for study from the Culture Collection of Algae, Indiana University, at the suggestion of Dr. Richard C. Starr. The alga is clearly not a member of *Borodinella* as Miller (1928) originally described it, because the chloroplasts are not axile with radiating arms, but, instead, hollow and cup-like, with varying degrees of peripheral fissuring; furthermore, the zoospores do *not* become spherical upon quiescence. Accordingly, the writers consider this culture to exemplify organisms in the genus *Tetracystis,* rather than *Borodinella.*

Tetracystis dissociata has many attributes in common with *T. isobilateralis, T.*

aggregata, and *T. illinoisensis* but differs significantly from *T. illinoisensis,* which it most closely resembles, in: (1) the absence of contractile vacuoles in the vegetative cells; (2) zoospores which have flagella longer than the cellular body length; (3) insignificant cell wall thickening at 3 months on BBM agar; and (4) the apparent absence of sexual reproduction.

Tetracystis excentrica sp. nov. ("Opera") (Fig. 1–12, 101–102)

Cellulae vegetativae iuvenes ellipsoideae, in cellulas vegetativas sphericas 16 μ diam. celeriter maturescentes. Incrassationes, membranae internales unipolares bipolaresque (1–1/2—4 × 3/4 μ) in culturis duarum habdomadum aetate notabiles. Chloroplastus parietalis, subsolidus, punctatus dilute viridis; fissuris nullis. Areola cytoplasmica internalis magna liquida in cellulis vegetativis maturis observabiles. Chloroplastus unicum chloroplastum parietalem, unicum pyrenoideum equatoriale, stigma maxime anterius, hemisphericis praeditum, continens. Cellulae in incrementi periodo immobili flavo-aurantiae factae. Duae vel multae vacuolae contractiles in cellulis vegetativis maturis uninucleatis.

Reproductio sexualis per gametas isogameticas a zoosporis morphologicaliter indis-vegetativarum, maturarum a membrana cellulae parentis mox dissociatarum, directe efficientem. Tetrades ad complexus formandos non associatae. Reproductio asexualis etiam per zoosporas aplanosporasque. Zoosporae cylindricae, 3 × 10 μ postice satis amplificiatae, antice acuminatae; nucleum posteriorem duas vacuolas contractiles, chloroplastum parietalem, unicum pyrenoideum equatoriale, stigma maxime anterius, minutum lineare et duo flagella longitudine aequa paululo breviora quam longitudo corporis cellulae habentes.

Reproductio sexualis per gametas isogameticas a zoosporis morphologicaliter indistinguibiles. Plasmogamia 3–5 minutis post copulationem perfecta. Zygotum membranam levem habens, a cellulis vegetativis indistinguible, fructus germinationis zygoti ignoti.

Culturae duarum hebdomadum aetate in medio "agar" basali dilute-virides; superficies coloniae levis (magnificatione sex et 12 plo); coloniae magnificatione nulla humidae ad nitidas.

Origo: e solo granitico distante 7 milia passuum ab loco Evergreen, Colorado dicto, m. Aug. 1961.

Young vegetative cells ellipsoidal, rapidly maturing into spherical vegetative cells 16 μ in diameter. Significant, internal unipolar and bi-polar wall thickenings (1½–4 × 3/4 μ) in 2-week-old cultures. Chloroplast parietal, somewhat massive, punctate, light-green; fissures absent. Large, clear internal cytoplasmic area observable in mature vegetative cells. Chloroplast containing a single, excentric, ellipsoidal pyrenoid (long axis, 3–4 μ), with 2 hemispherical starch grains. Cells becoming yellow-orange in stationary phase of growth. Two-to-many contractile vacuoles present in mature, uninucleate vegetative cells.

Asexual reproduction by cell division giving rise directly to tetrads (rarely to diads) of vegetative cells which soon dissociate from parent cell wall at maturity.

Tetrads not associated to form complexes. Asexual reproduction also by zoospores and aplanospores. Zoospores cylindrical, 3 × 10 μ, somewhat broadened posteriorly and pointed anteriorly; with a posterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single equatorial pyrenoid, a very anterior, minute, linear stigma, and 2 flagella of equal length, slightly shorter than cell body length.

Sexual reproduction by isogamous gametes indistinguishable morphologically from zoospores. Plasmogamy completed within 3–5 min after pairing. Zygote smooth-walled, indistinguishable from vegetative cells, the products of zygote germination unknown.

Two-week-old cultures on basal agar medium, light-green; colony surface smooth (6 and 12 × magnification); colonies moist to shiny macroscopically.

Source: granitic soil 7 miles west of Evergreen, Colorado, August, 1961.

The soil collection from which this organism was isolated was made by Dr. and Mrs. R. Malcolm Brown of Pampa, Texas, August, 1961. *Tetracystis excentrica* is closely related to 3 other species of *Tetracystis,* namely *T. intermedium, T. texensis,* and *T. pulchra.* All 4 of these species of *Tetracystis* have in common vegetative cells with an ellipsoidal pyrenoid surrounded by 2 starch grains. The pyrenoid is also excentric in the cells.

Tetracystis excentrica is characterized by several salient features: (1) yellow-orange colonies on BBM agar at 3 months; (2) 2-to-many contractile vacuoles in the vegetative cells (when more than two are present, they are in the peripheral protoplasm of the cell rather than in the perinuclear region); (3) daughter cells of the tetrad which dissociate soon after wall formation is complete; and (4) a smooth colony surface on BBM at 2 weeks.

Tetracystis excentrica is the only species in which the product of isogamous plasmogamy (Fig. 4–11) is a smooth-walled zygote, indistinguishable from the vegetative cell, at least during some part of its ontogeny. A diplobiontic cycle may be operative within this species; however, more intensive study will be needed in order to prove this hypothesis. After a culture on BBM agar has grown for about 2–3 weeks, several very large cells (20–35 μ in diameter) with several pyrenoids have been observed (Fig. 12). It is entirely possible that these cells may represent the zygote in a later phase of its ontogeny. The giant cells are multinucleate. Unequal, uninucleate segments of the protoplast frequently are formed and break away from the main portion (Fig. 12). Whether or not these uninucleate segments abort or live is unknown at present.

One of the most beautiful and striking occurrences of sexual reproduction among the Chlorophyta in the writers' experience has been observed in *T. excentrica.*[1] If one takes an actively growing culture and places a loopful of inoculum into a hanging drop culture (in distilled water) in the afternoon of a given day, he will

[1] The complete process, photographed at 5-sec intervals, is shown in Fig. 4–11.

find abundant pairing of gametes the following morning. Following pairing, the process of plasmogamy is extremely rapid, once it starts. About 5–15 min after pairing, a cytoplasmic bridge will be formed between the 2 gametes. Then, in an instant (5–30 sec), the protoplast of one gamete will unite with that of the other, and the gametic walls will be shed. The daughter chloroplasts seem to unite (Fig. 5–9), but the 2 pyrenoids of the zygote have not been observed to fuse. The fate of the zygote is unknown because, as it enlarges, it becomes indistinguishable from the vegetative cells.

Tetracystis illinoisensis. (R–3–3) (Figs. 53–56, 85–86)

Cellulae vegetativae iuvens ellipsoidales ad ovatas, in cellulas vegetativas ovatas ad sphericas 12–14 μ diam. maturescentes. Incrassationes membranae cellulae in culturis duarum hebdomadum aetate non notabilis, post tres menses, autem, incrassationes (5 μ) internae manifestae unipolases bipolaresque adsunt. Chloroplastus solidus paucissimas fissuras praebens, unicum pyrenoideum paululum excentricum forma irregulare (4–5 μ diam.) et multas amyli micas continens. Cellulae in incrementi periodo immobili (tribus mensibus) virides. Cellulae vegetativae uninucleatae duas vacuolas contractiles habens.

Reproductio asexualis per cellulae divisionem in diades tetradesque cellularumfiliarum vegetativarum quae maturae primum in membrana cellulae parentis includentur et eis adhaerent. Diades tetradesque ad complexus formandos non aggregatae. Reproductio asexualis etiam per zoosporas aplanosporasque. Zoosporae cylindricae 3–4 × 8–10 μ, nucleum posteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum pyrenoideum equatoriale, stigma equatoriale ad paululo anterius, et duo flagella paululo breviora quam longitudo corporis cellulae habentes.

Reproductio sexualis non observata.

Culturae duarum hebdomadum aetate in medio "agar" basali dilute virides. Superficies coloniae homogenea (ad minute granulosa) magnificatione sex et 12 plo; coloniae magnificatione nulla opaconitidae.

Origo: e patina "Petri" medii culturae aeri ex automobili exposita, in loco distante 30 milia passuum ab oppido Effingham, Ill. dicto, m. Sept. 1962.

Young vegetative cells ellipsoidal to ovoidal, maturing into ovoidal to spherical vegetative cells 12–14 μ in diameter. Cell wall thickening insignificant in cultures 2 weeks old, but prominent internal, unipolar and bipolar thickenings (5 μ) present at 3 months. Chloroplast massive with very few fissures, containing a single, slightly excentric, irregularly shaped pyrenoid (4–5 μ in diameter) with many starch grains. Cells green in the stationary phase of growth (3 months). Uninucleate vegetative cells with 2 contractile vacuoles.

Asexual reproduction by cell division into diads and tetrads of daughter vegetative cells which at maturity initially remain enclosed by and adhere to the parent cell wall. Diads and tetrads not aggregating to form complexes. Asexual reproduction also by zoospores and aplanospores. Zoospores cylindrical 3–4 × 8–10 μ, with a posterior nucleus, 2 anterior contractile vacuoles, parietal chloroplast, a single

equatorial pyrenoid, and equatorial to slightly anterior stigma, and 2 flagella which are slightly shorter than cell body length.

Sexual reproduction not observed.

Two-week-old cultures on basal agar medium, light green; colony surface homogeneous (to minutely granular) at 6 and 12 × magnification; colonies dull-shiny macroscopically.

Source: from Petri dish of culture medium exposed to air from an automobile, 30 miles east of Effingham, Illinois, September, 1962.

This organism was kindly provided to the writers by Misses Pat Walne and Elenor Cox who collected it from the air by exposing a Petri plate to sterile BBM agar from a moving automobile 30 miles east of Effingham, Illinois, September, 1962.

Tetracystis illinoisensis has many attributes in common with *T. isobilateralis, T. aggregata,* and *T. dissociata;* however, it differs significantly from *T. dissociata,* which it most closely resembles, in: (1) the presence of 2 contractile vacuoles in the vegetative cell; (2) zoospores with flagella slightly shorter than the cellular body length; and (3) cell walls with unipolar and bipolar thickenings (up to 5 μ) on BBM agar at 3 months.

Like *T. dissociata, T. illinoisensis* is characterized by the presence of tetrads (mostly tetrahedral) which never aggregate to form complexes. Unlike *T. dissociata, T. illinoisensis* undergoes a delayed tetrad formation; this accounting for the small population of tetrads in cultures on BBM agar at 2 weeks.

Tetracystis isobilateralis sp. nov. (A_6–2–3) (Fig. 41–48, 81–82)

Cellulae vegetativae maturae sphericae ad subsphericas, 18–19 μ diam. Incrassatio membranae cellulae non notabilis in culturis duarum hebdomadum aetate, varians, autem, ab 2 ad 3 μ in culturis trium mensium aetate. Chloroplastus solidus quasi macuolosus atque dilute, viridis, totum cellulae lumen, area perinucleari excepta, complens; fissuris profundis nullis. Chloroplastus unicum pryenoideum centrale magnum 5–7 μ diam., forma irregulare, multis amyli lamellis circumdatum, habens. Cellulae in incrementi periodo immobili semper virides. Vacuolae contractiles in cellulis vegetativis uninucleatis nullae.

Reproductio asexualis per cellulae divisionem diades tetradesque cellularum vegetativarum, maturarum membranae parentis conferte associatarum, directe efficientem. Complexus unius vel aliquot tetradum frequentes. Reproductio asexualis etiam per zoosporas aplanosporas bipartitione successiva protoplasti multinucleati formatas. Zoosporae ovatae 4–6 × 8–10 μ, nucleum posteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unum ad aliquot pyrenoidea equatorialia, stigma equatoriale ad paululo anterius et duo flagella longitudine aequa, 1½ plo longiora quam longitudo corporis habentes.

Reproductio sexualis per gametas isogamicas, a zoosporis, nisi quod paululo minoribus, morphologicaliter indistinguibiles. Zygotum echinatum, 15–20 μ diam., divisionem instantem subiens, quattuor cellulas vegetativas efficiens.

Culturae duarum hebdomadum aetate in medio "agar" basali dilute virides; superficies coloniae aspera (minute scabellata) magnificatione sex et 12 plo; coloniae magnificatione nulla opaconitidae aut siccae.

Origo: e solo a loco Blackland Prairie Region of Williamson County, Texas, dicto, m. July 1960.

Mature vegetative cells spherical to subspherical, 18–19 μ in diameter. Cell wall thickening insignificant in 2-week-old cultures but ranging from 2 to 3 μ in cultures 3 months old. Chloroplast massive, somewhat mottled and light green, filling the entire cell lumen except for the perinuclear area; deep fissures absent. Chloroplast with a single, large, central pyrenoid, 5–7 μ in diameter, irregular shaped and surrounded by many starch plates. Cells remaining green in the stationary phase of growth. Contractile vacuoles absent in uninucleate vegetative cells.

Asexual reproduction by cell division giving rise directly to diads and tetrads of vegetative cells which are intimately associated with the parent wall at maturity. Complexes of 1 to several tetrads frequent. Asexual reproduction also by zoospores and aplanospores formed by successive bipartition of a multinucleate protoplast. Zoospores ovoid 4–6 $\times$ 8–10 μ; with a posterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, 1-to-several equatorial pyrenoids, equatorial-to-slightly anterior stigma, and 2 flagella of equal length, 1½ times body length.

Sexual reproduction by isogamous gametes, indistinguishable morphologically from the zoospores except for their slightly smaller size. Zygote echinate, 15–20 μ in diameter, undergoing immediate division giving rise to 4 vegetative cells.

Two-week-old cultures on basal agar medium, light-green; colony surface rough (minutely scabellate) at 6 and 12 $\times$ magnification; colonies dull-shiny or dry macroscopically.

Source: soil from Blackland Prairie Region of Williamson County, Texas, July, 1960.

This species was provided to the writer by Mrs. LaVerne Johnston who originally isolated it. This alga is especially striking in the mode of formation of isobilateral tetrads of daughter cells (i.e., all 4 daughter cells can be observed in the same focal plane).

Most tetrads originate through an intervening diad stage, but tetrads also can be formed directly in *T. isobilateralis*. The sequence of events leading to the formation of tetrads as it commonly occurs in *T. isobilateralis* is shown in Text-fig. 3 (see page 63).

Tetracystis aggregata, T. dissociata, and *T. illinoisensis* have many attributes in common with *T. isobilateralis*; however, the latter is characterized by a larger mature vegetative cell, which ranges from 18–19 μ in diameter, and by the more abundant production of isobilateral tetrads.

Tetracystis isobilateralis is particularly well delineated ultrastructurally in that it is the only presently known species of *Tetracystis* with giant, branched mitochondria.

Tetracystis pampae sp. nov. (Pampa) (Fig. 57–64, 105–106)

Cellulae vegetativae iuvenes ellipsoideae, in cellulas vegetativas sphericas ad subsphericas 14 μ diam. maturescentes. Incrassatio membranae cellulae non notabilis in culturis duarum hebdomadum usque ad eas trium mensium aetate. Chloroplastus solidus, amylo impletus, aliquot fissuras non profundas praebens, pyrenoideum vix excentricum ad centrale, forma irregulare (4 μ diam.), multis amyli micis circumdatum, continens. Cellulae in incrementi periodo immobili (tribus mensibus) semper nitido-virides. Cellulae vegetativae uninucleatae duas vacuolas contractiles continentes.

Reproductio asexualis per divisionem cellulae in diades atque plerumque tetrades in periodo incrementi tardo-logarithmico. Diades tetradesque cellularum filiarum maturarum membranae cellulae parentis firme adhaerentes. Tetrades complexus saepe formantes. Reproductio asexualis etiam per zoosporas aplanosporasque. Zoosporae cylindricae, 4–8 μ, nucleum posteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum pyrenoideum equatoriale stigma maxime anterius et duo flagella longitudine, aequa, quasi aequa longitudine corpori cellulae habentes.

Reproductio sexualis non observata.

Culturae duarum hebdomadum aetate in medio "agar" basali atrovirides; superficies coloniae paululum aspera rugosaque magnificatione sex et 12 plo; coloniae magnificatione nulla siccae. Materia propria brunnea e cellulis in "agar" sub duabus ad tres hebdomades aetate diffusa.

Origo: e solo a florum areola in loco Pampa, Texas, dicto, m. Apr. 1961.

Young vegetative cells ellipsoidal, maturing into spherical to subspherical vegetative cells 14 μ in diameter. Cell wall thickening insignificant in cultures from 2 weeks to 3 months old. Chloroplast massive and starchy, with few shallow fissures. Chloroplast containing a very slightly excentric to central irregularly shaped pyrenoid (4 μ in diameter), surrounded by many starch grains. Cells remaining bright green in stationary phase of growth (3 months). Uninucleate vegetative cells containing 2 contractile vacuoles.

Asexual reproduction by cell division into diads and mostly tetrads in late log phase of growth. Diads and tetrads of daughter cells at maturity strongly adherent to parent cell wall. Tetrads often forming complexes. Asexual reproduction also by zoospores and aplanospores. Zoospores cylindrical, 4–8 μ, with a posterior nucleus, 2 anterior contractile vacuoles, parietal chloroplast, single equatorial pyrenoid, a very anterior stigma, and 2 flagella of equal length, about equal to the cell body length.

Sexual reproduction not observed.

Two-week-old cultures on basal agar medium, dark-green; colony surface slightly rough and rugose at 6 and 12 $\times$ magnification; colonies dry macroscopically. Characteristic brown substance diffusing from cells into agar by 2–3 weeks.

Source: soil from flower bed, Pampa, Texas, April, 1961.

Tetracystis pampae has several attributes in common with *T. aggregata* and *T. isobilateralis*, its closest allies; however, *T. pampae* is distinct from all species of

Tetracystis in the production of a characteristic brown substance diffusing from the cells into BBM agar by 2–3 weeks. *Tetracystis pampae* is distinguished from its 2 closest allies in having: (1) mature vegetative cells, 14–16 μ in diameter, which are slightly ellipsoidal; (2) delayed tetrad production; (3) a rugose colony surface at 2 weeks on BBM; (4) zoospores with a very anterior stigma; (5) insignificant cell wall thickening at 3 months on BBM agar; and (6) the absence of sexual reproduction.

Tetracystis pampae is distinct from all other species of *Tetracystis* in the ultrastructure of its pyrenoid and chloroplast as will be discussed in a subsequent section. In addition, certain physiological tests show *T. pampae* to be a very distinct *Tetracystis* species.

Tetracystis pulchra sp. nov. (Sweet) Fig. 49–52, 97–98)

Cellulae sphericae iuvenes ellipsoideae, in cellulas vegetativas paululum ellipsoideas ad sphericas 12–14 μ diam. maturescentes. Incrassatio bipolaris membranae cellularum (ad 1–3 μ in culturis duarum hebdomadum aetate ad 5 μ trium mensium). Chloroplastus parietalis, paucissimas fissuras praebens; pyrenoideum ellipsoideum axe longo 3–4 μ, duabus amyli micis circumdatum, excentricum. Cellulae nitidoaurantiae in incrementi periodo immobili factae. Duae vacuolae contractiles in cellulis vegetativis uninucleatis.

Reproductio asexualis per cellulae divisionem (plerumque) tetrades cellularum-filiarum vegetativarum, maturarum membrane cellulae-parentis firme adhaerentium directe efficientem. Tetrades ad complexus satis magnos formandos aggregatae. Reproductio asexualis etiam per zoosporas aplanosporasque. Zoosporae cylindricae, 2 × 7 μ, nucleum posteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum pyrenoideum minutum equatoriale, stigma maxime anterius minutissimum et duo flagella longitudine aequa, longitudine quasi aequa corpori cellulae aut paululo longiora habentes.

Reproductio sexualis non observata.

Culturae duarum hebdomadum aetate in medio "agar" basali modice viriles aut paululum aurantias; superficies coloniae homogenea ad minute granulosam, magnificatione sex et 12 plo; coloniae magnificatione nulla siccae.

Origo: plantae e solo isolatae e loco Austin, Texas, dicto., m. Jul. 1962.

Young vegetative cells ellipsoidal, maturing into slightly ellipsoidal to spherical vegetative cells 12–14 μ in diameter. Cells with bipolar wall thickening (to 1–3 μ in 2-week-old cultures, to 5 μ in 3-month-old cultures). Chloroplast parietal with very few fissures; pyrenoid ellipsoidal, long axis 3–4 μ, surrounded by 2 starch grains, and excentric. Cells becoming bright-orange in stationary phase of growth. Two contractile vacuoles present in the uninucleate vegetative cells.

Asexual reproduction by cell division giving rise directly (mostly) to tetrads of daughter vegetative cells strongly adherent to the parent cell wall at maturity. Tetrads aggregated to form moderately large complexes. Asexual reproduction also by zoospores and aplanospores. Zoospores cylindrical, 2 × 7 μ, with a posterior

nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single, minute, equatorial pyrenoid, a very anterior and very minute stigma, and 2 flagella of equal length, about equal to slightly longer than cell body length.

Sexual reproduction not observed.

Two-week-old cultures on basal agar medium, green to slightly orange; colony surface homogeneous to minutely granular at 6 and 12 × magnification; colonies dry macroscopically.

Source: isolated from garden soil, exact origin of which is unknown. Austin, Texas, July, 1962.

This organism, herein described as a new species of *Tetracystis*, was isolated in July, 1962, by Mr. Charles Sweet.

Tetracystis pulchra is closely related to *T. excentrica*, to *T. texensis*, and, more especially, to *T. intermedium*. Two attributes distinguish *T. pulchra* from *T. intermedium*: (1) the occurrence of a thickened cell wall in cultures of *T. pulchra* at 3 months on BBM agar; and (2) the presence of a homogeneous to minutely granular colony.

Tetracystis texensis sp. nov. (Mx_2–c) (Fig. 19–24, 95–96)

Cellulae vegetativae iuvenes ellipsoideae, in cellulas vegetativas sphericas 14–15 μ diam. celeriter maturescentes. Incrassatio membranae cellulae usque 3–4 μ in culturis duarum hebdomadum aetate, in culturis vetustioribus non aucta. Chloroplastus parietalis tenuisque saepe striatus et profunde lobatus, pyrenoideum maxime excentricum ellipsoideum (axe longo 3–4 μ) duabus micis amyli hemisphericis praeditum, continens. Cellulae in incrementi periodo immobili (tribus mensibus) flavo-brunneae factae. Vacuolae contractiles in cellulis vegetativis uninucleatis nullae.

Reproductio asexualis per cellulae divisionem tetrades octadesque directe efficientem. Aggregationes tetradum membranis cellulae parentis conferte associatae, octades, autem, minus conferte associatae. Octades, saepius tetrades, ad complexus cellularum multarum formandos cohaerentes. Reproductio asexualis raro quoque per zoosporas aplanosporasque. Zoosporae 3 × 10 μ nucleum posteriorem, duas vacuolas contractiles anteriores, chloroplastum parietalem, unicum pyrenoideum paululo anterius, stigma anterius et duo flagella longitudine aequa, corpori cellulae aequa aut paululo breviora.

Reproductio sexualis non observata.

Culturae duarum hebdomadum aetate in medio basali atro-ad nitido-virides; superficies coloniae irregulariter aspera maculosaque (minute botryoidea) magnificatione sex et 12 plo; coloniae a humidis ad siccas variantes magnificatione nulla.

Origo: e soli examplo a loco Pilot Knob, Travis County, Texas dicto, m. Mai. 1961.

Young vegetative cells ellipsoidal, rapidly maturing into spherical vegetative cells 14–15 μ in diameter. Cell wall thickening up to 3–4 μ in 2-week-old cultures, thickening not increasing in older cultures. Chloroplast parietal and thin, often striate and deeply lobed. Chloroplast containing a very excentric, ellipsoidal pyrenoid (long axis 3–4 μ), with 2 hemispherical starch grains. Cells becoming yellow-brown

in stationary phase of growth (3 months). Contractile vacuoles absent in the uninucleate vegetative cells.

Asexual reproduction by cell division giving rise directly to tetrads or octads. Tetrad aggregates intimately associated with parent cell walls, but octads less intimately associated. Octads, and more frequently, tetrads coherent to form complexes of many cells. Asexual reproduction infrequently also by zoospores and aplanospores. Zoospores 3 × 10 μ, with a posterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a slightly anterior, single pyrenoid, an anterior stigma, and 2 flagella of equal length, slightly shorter than equal to the cell body length.

Sexual reproduction not observed.

Two-week-old cultures on basal medium, dark-to-bright green; colony surface irregularly rough, mottled (minutely botryoid) at 6 and 12 × magnification; colonies range from moist to dry macroscopically.

Source: soil sample from Pilot Knob, Travis County, Texas, May, 1961.

This organism was kindly given to the writers by Dr. Karl Mattox who originally isolated it.

Tetracystis texensis belongs to a group of closely related *Tetracystis* species (*T. excentrica*, *T. intermedium*, and *T. pulchra*), all of which have similar pyrenoid types as discussed previously. However, *T. texensis* is readily differentiated from these species by having: (1) yellow-brown colonies on BBM agar at 3 months; (2) formation of octads of non-motile daughter cells in addition to tetrads; (3) infrequent and difficult-to-evoke zoospore formation; and (4) in lacking contractile vacuoles in the vegetative cells. In light of these considerations, *T. texensis* is herein described as a new species of the genus *Tetracystis*.

As noted in the Introduction, 3 algae previously described by other investigators as species of *Chlorococcum* have been investigated concurrently and comparatively with the species of *Tetracystis*, inasmuch as the writers hypothesized that the former 3 taxa might more appropriately be included in the genus *Tetracystis*. All the data obtained with the several methodologies used in this research have convinced us that, indeed, these 3 suspect *Chlorococcum* species should be transferred to *Tetracystis*. It should be noted in passing that Deason and Bold (1960) earlier had questioned whether these 3 taxa were, in fact, members of *Chlorococcum*. The amended and augmented characterizations of these taxa now follow.

Tetracystis aplanosporum (*Chlorococcum aplanosporum* Arce and Bold) comb. nov. (Fig. 65–72, 103–104)

Young vegetative cells ovoidal, maturing into spherical vegetative cells 18–27 μ in diameter. Cell wall thickening insignificant at 2 weeks but ranging from 2 to 3 μ in cultures 3 months old. Chloroplast massive with deep fissures and a large, clear, unilateral, cytoplasmic sinus observable in actively growing cultures. Chloroplast

containing a central irregularly shaped pyrenoid, 5–6 μ in diameter, surrounded by many starch grains. Cells remaining green in stationary phase of growth (3 months). From 2 to 6 large contractile vacuoles always present in actively growing reproductive (zoospores and aplanospores) and non-motile uninucleate vegetative cells (Fig. 66, arrow).

Asexual reproduction by cell division giving rise exclusively, directly to tetrads of daughter vegetative cells which at maturity are loosely associated with parent cell wall. Tetrads not characteristically aggregating to form complexes. Asexual reproduction also by zoospores and aplanospores formed by a series of successive bipartitions. Zoospores broadly cylindrical (3–6 $\times$ 8–16 μ) with an anterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single slightly posterior pyrenoid, equatorial to slightly anterior stigma, and 2 equal flagella approximately the length of the cellular body.

Sexual reproduction not observed.

Two-week-old cultures on basal agar medium, light green; colony surface rough, minutely vermiform at 6 and 12 $\times$ magnification; colonies moist macroscopically.

Source: Indiana University Culture Collection No. 773 as *Chlorococcum aplanosporum* Arce and Bold.

What appear superficially to be tetrads of aplanospores are, in fact, tetrads of non-motile daughter cells which have not originated from zoosporic antecedents. For this reason, *Chlorococcum aplanosporum* is herein transferred to the genus *Tetracystis*.

Tetracystis aplanosporum and *T. aeria* are the only 2 presently known species of *Tetracystis* in which the zoospores have anterior nuclei. However, *T. aplanosporum* is clearly distinguishable from *T. aeria* by: (1) its larger mature vegetative cells, 19–27 μ in diameter; (2) 2–4 large contractile vacuoles in the vegetative cell; (3) dissociation of tetrads at the tetrad level; (4) zoospores with flagella equal to the cell body length; (5) an equatorial-to-slightly anterior stigma in the zoospore; (6) thinner cell walls at 2 weeks and 3 months on BBM agar; and (7) a rough colony surface which is minutely vermiform on BBM agar at 2 weeks.

Tetracystis aplanosporum clearly is one of the most distinct species of *Tetracystis* on the basis of both light microscopy and its physiology. This statement is further supported by ultrastructural and immunochemical evidence which will be presented in later sections.

Tetracystis intermedium (*Chlorococcum intermedium* Deason and Bold) comb. nov. (Fig. 73–78, 99–100)

Young vegetative cells ellipsoidal, maturing into vegetative cells 12–20 μ in diameter; cell wall not markedly thickened even in cultures 2 months old. Chloroplast thin and parietal with a few shallow fissures. Chloroplast containing a single, excentric, ellipsoidal pyrenoid 2 μ in diameter, surrounded by 2 starch grains. Cells

becoming bright-orange in stationary phase of growth (3 months). Uninucleate vegetative cells containing 2 contractile vacuoles.

Asexual reproduction by cell division giving rise to diads and (mostly) to tetrads of vegetative cells which at maturity are strongly adherent to the parent cell wall; several tetrads aggregating to form simple complexes. Asexual reproduction also by zoospores and aplanospores formed by rapid successive bipartitions. Zoospores 2.5–9.5 μ, with a posterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single equatorial pyrenoid, a very anterior stigma, and 2 flagella which are slightly longer than body length.

Sexual reproduction not observed.

Two-week-old cultures on basal medium, green, becoming slightly orange; colony surface rough and granular at 6 and 12 $\times$ magnification; colonies moist to dull-shiny macroscopically.

Source: Indiana University Culture Collection No. 974 *Chlorococcum intermedium* Deason and Bold.

As noted above, this organism, as well as *T. aplanosporum* and *T. tetrasporum*, was originally described as a *Chlorococcum* species. The basis for Deason and Bold considering this alga to be a *Chlorococcum* species was that this organism did not undergo vegetative cell division *sensu* Herndon. Deason and Bold considered the timing of cytokinesis and wall formation especially significant. Each cytokinesis followed by intervening cell-wall deposition was considered by them as true vegetative cell division *sensu* Herndon. If rapidly occurring divisions resulted in a multinucleate protoplast in which cell-wall deposition was delayed until all cytokinesis had been completed, the organism in question was classified by them in the Chlorococcales. Such divisions of a multinucleate protoplast were considered analagous to those in zoosporogenesis by Deason and Bold.

In view of the writers' emphasis that in the order Chlorosphaerales, there are formed, by cell division, non-motile daughter vegetative cells which lack the potential for motility, *Chlorococcum intermedium* has been transferred from the Chlorococcales to the genus *Tetracystis* of the Chlorosphaerales. In addition, ultrastructural evidence seems to support the validity of this transfer in that ultrastructural differences in the ontogeny of zoospore and daughter vegetative cells are striking.

Tetracystis intermedium belongs to a group of closely related *Tetracystis* species, namely, *T. texensis*, *T. excentrica*, and *T. pulchra*. *Tetracystis intermedium* differs from *T. pulchra*, which it mostly closely resembles, in: (1) the absence of cell wall thickenings on BBM at 3 months; and (2) in its rough colony surface.

Tetracystis tetrasporum (*Chlorococcum tetrasporum* Arce and Bold) comb. nov. (Fig. 79–80, 93–94)

Young vegetative cells ellipsoidal, maturing into spherical vegetative cells 15–

18 μ in diameter. Cell wall thickening insignificant at 2 weeks and also at 3 months. Chloroplast massive with few shallow fissures. Chloroplast containing a central-to-slightly excentric, almost spherical pyrenoid, 4–5 μ in diameter, surrounded by many closely packed starch grains. Cells remaining green in stationary phase of growth. Uniculeate vegetative cells contain 2 contractile vacuoles.

Asexual reproduction by cell division giving rise to tetrads of daughter vegetative cells, only late in growth phase (after 1 month). Tetrads strongly adherent to parent cell wall. Several tetrads adhere at maturity to form cellular complexes. Asexual reproduction also by zoospores and aplanospores. Zoospore production prolonged for at least 2 weeks following inoculation in basal agar medium. Zoospores 4 × 10 μ, with a posterior nucleus, 2 anterior contractile vacuoles, a parietal chloroplast, a single equatorial pyrenoid, a large anterior stigma, and 2 flagella of equal length which are slightly shorter than the cellular body length.

Two-week-old cultures on basal medium, green; colony surface smooth and homogeneous at 6 and 12 × magnification; colonies macroscopically shiny. Four-to-six week old cultures on basal medium, green; colony surface rough and granular (due to tetrad production) at 6 and 12 × magnification; colonies macroscopically moist-to-dry.

Source: Indiana University Culture Collection of Algae No. 780 as *Chlorococcum tetrasporum* Arce and Bold.

Tetracystis tetrasporum can be especially deceptive and difficult to identify as a species of *Tetracystis* because tetrad formation occurs only very late after inoculation upon slants of BBM agar (i.e., 4–6 weeks). This very much delayed tetrad production is peculiar to *T. tetrasporum* among the presently known species of *Tetracystis*. During the initial period of growth (2–4 weeks), prolonged zoosporogenesis occurs. Another closely related species, *T. illinoisensis*, produces tetrads late after inoculation, not so late, however, as *T. tetrasporum*. *Tetracystis illinoisensis*, furthermore, is differentiated from *T. tetrasporum* in that the latter undergoes a prolonged period of zoosporogenesis prior to tetrad formation.

Therefore, in view of the occurrence of non-motile daughter cells in tetrads, *Chlorococcum tetrasporum* is herein transferred from *Chlorococcum* to *Tetracystis*.

KEY TO THE CURRENTLY KNOWN SPECIES OF *TETRACYSTIS* BROWN AND BOLD

1. Zoospore with anterior nucleus 2
1. Zoospore with posterior nucleus 3
   2. Mature vegetative cells 18–19 μ in diameter; 2-to-many contractile vacuoles in vegetative cells; tetrad dissociation at the tetrad level, forming no cellular complexes ..... *T. aplanosporum* comb. nov.
   2. Mature vegetative cells 14–16 μ in diameter; 2 contractile vacuoles occasionally present in vegetative cells; tetrads cohering to form cellular complexes ..... *T. aeria* sp. nov.

3. Pyrenoid more or less centrally located in vegetative cell and surrounded by many starch grains; pyrenoid spherical to irregular 4
3. Pyrenoid ellipsoidal, with only 2 starch grains; excentric in the vegetative cell 9
4. Colonies on BBM agar smooth and homogeneous at 2 weeks 5
4. Colonies rough on BBM agar at 2 weeks 7
5. Cell wall thickenings insignificant both at 2 weeks and 3 months on BBM agar 6
5. Uni- and bipolar cell wall thickenings (up to 5 μ, present in cultures on BBM agar at 3 months ..... *T. illinoisensis* sp. nov.
6. Tetrad production delayed until very late in development (after 1 month on BBM agar) ..... *T. tetrasporum* comb. nov.
6. Tetrad production occurring within 1–2 weeks after inoculation on BBM agar ..... *T. dissociata* sp. nov.
7. Mature vegetative cells slightly ellipsoidal, 12–14 μ; zoospores with a very anterior stigma; colony surface rugose; cell wall thickening insignificant at 3 months on BBM agar; sexual reproduction not observed; delayed tetrad production; a distinct, brown substance secreted into BBM agar at 2–4 weeks ..... *T. pampae* sp. nov.
7. Mature vegetative cells mostly spherical; zoospores with an equatorial-to-slightly anterior stigma; cell wall thickening 2–4 μ at 3 months on BBM agar; sexual reproduction present 8
8. Mature vegetative cells 15–16 μ in diameter, with 2 contractile vacuoles; colonies coarsely granular at 2 weeks on BBM agar; deep radiating fissures often present in chloroplast ..... *T. aggregata* sp. nov.
8. Mature vegetative cells 18–19 μ in diameter; contracile vacuoles absent in vegetative cells; colony surface minutely scabellate on BBM agar at 2 weeks; isobilateral tetrads prominent at 2 weeks ..... *T. isobilateralis* sp. nov.
9. Color of colony bright-orange to red-orange at 3 months on BBM agar 10
9. Color of colony otherwise 11
10. Colony surface rough on BBM agar at 2 weeks; cell wall thickenings insignificant (1–2 μ) at 3 months on BBM agar .... *T. intermedium* comb. nov.
10. Colony surface homogeneous to minutely granular on BBM agar at 2 weeks; cell wall thickenings up to 5 μ at 3 months on BBM agar ..... *T. pulchra,* sp. nov.
11. Color of colony yellow-orange at 3 months on BBM agar; colony surface smooth; daughter cells in tetrads dissociate early; 2-to-many peripheral, contractile vacuoles in vegetative cell; sexual reproduction observed; zygote smooth-walled ..... *T. excentrica* sp. nov.

11. Color of colony yellow-brown at 3 months on BBM agar; colony surface rough; numerous octads of non-motile daughter cells produced in addition to tetrads; contractile vacuoles absent from vegetative cells; zoospore formation infrequent and difficult to evoke ..... *T. texensis* sp. nov.

D. PHYSIOLOGICAL ATTRIBUTES IN *Tetracystis*

In recent years, unicellular and other chlorophycean algae have been treated taxonomically, in part, on the basis of physiological attributes (Deason and Bold, 1960; Mattox and Bold, 1962; Bold and Parker, 1962; Chantanachat and Bold, 1962; and Bischoff and Bold, 1963).

Physiological and other, so-called supplementary, attributes have provided extremely useful data which help determine the taxonomic affinities of certain species and genera. In the writers' experience, as well as that of previous investigators, it has been found indispensable to run duplicate cultures when performing a given physiological test, and to repeat each test at least one or more times.

Most of the physiological tests were performed before the writers had isolated all but two of the *Tetracystis* species discussed in this report. Accordingly, data regarding the last 2 species isolated, namely, *T. illinoisensis* and *T. pulchra*, regretfully will not be included in summaries of several of the physiological tests. Since these 2 species, as well as the other species of *Tetracystis*, are easily characterized on morphological grounds, the physiological data are not necessary for their differentiation but may have served to amplify and to augment our knowledge based on morphological attributes.

The data on these physiological attributes are summarized in Tables 1–6 and in the sections immediately following.

Effects of carbon compounds

Bold and Parker (1962), in an investigation of supplementary attributes in classifying species of *Chlorococcum,* reported taxonomically useful results when the inorganic medium was supplemented with a variety of sugars, acetate, and pyruvate. It should be noted that in their procedures, the carbon compounds were autoclaved after they had been dissolved in the inorganic medium. In the present investigation, xylose, ribose, fructose, glucose, arabinose, and sodium acetate were separately dissolved in de-ionized water and treated in 2 different ways: in one procedure, these fractions were Seitz-filtered, and in another, they were autoclaved at 15 p.s.i. for exactly 15 min. In both instances, these fractions were added aseptically to aliquots of sterile, cooled, liquid BBM in order to make a total concentration of 0.75% for each carbon source. The culture tubes had been rinsed 3 times in distilled water followed by 3 rinses in de-ionized water, plugged, and sterilized.

Axenic cultures of *Tetracystis* were inoculated into quadruplicate culture tubes containing 20 ml of BBM enriched with a given carbon source. A homogeneous inoculum was prepared as previously described (page 9), and 5 drops were aseptically transferred to each tube of the quadruplicate set by sterile Pasteur pipettes.

One set each of the autoclaved and Seitz-filtered media supplemented with the various carbon sources was placed in the light under standard conditions. A duplicate set of each was stored in darkness at the same temperature. After 14 days, cultures in the light were appraised for amount of growth. Cultures placed in darkness were removed 2 months after inoculation and examined for evidence of facultative heterotrophy. These same cultures were then placed in the light for 2 weeks under standard conditions, and the growth (recovery) in light, following a 2-month dark period, was noted and recorded. Growth was estimated visually in comparison with a set of standards, designated previously as "Excellent", "Good", "Fair", "Trace" and "None".

TABLE 1. *Growth of Tetracystis species in BBM supplemented with various carbon sources (at 0.75%) in light under standard conditions for 2 weeks (A = autoclaved; B = Seitz-filtered)*

Type of sterilization	Control (BBM) A	Xylose B	Xylose A	Ribose B	Ribose A	Arabinose B	Arabinose A	Glucose B	Glucose A	Fructose B	Fructose A	Na-acetate B	Na-acetate A
T. aeria (C-6)	E[a]	E	N	F	F	G	G	E	E	G	G	F	F
T. aeria (Pa-3)	G	F	F	F	F	G	G	E	E	G—G		F	F
T. isobilateralis	E	E	G	F	F	G	G	E	G	G+G		F	F
T. aggregata	E	G	F	F	N	G	G	G	G	G+F		F—	N
T. dissociata	G	G	G	T	N	F	F	G	G	F	F	F—	N
T. illinoisensis	G	G	N.D.	T	N.D.	G	N.D.	G	N.D.	G	N.D.	F+	N.D.
T. pampae	E	E	E	E—	E	E	E	E	E	E	E	E	E
T. aplanosporum	E	E	E	E—	E	E	E	E	E	E	E	F—	F
T. intermedium	E	E	E	G	F	E	E	E	E	E	E	F	F
T. tetrasporum	E	G	G	G	F	E	G	G	G	E	G	E	E
T. texensis	T	F	N.D.	N	N.D.	F	N.D.	T	N.D.	T	N.D.	N	N.D.
T. excentrica	G	G	G	F	F	F	F	G	G	G	G	N	N
T. pulchra	E	G	N.D.	F	N.D.	G	N.D.	G	N.D.	G	N.D.	F	N.D.

[a] E, growth excellent; G, good; F, fair; T, trace; N, no growth; N.D., no data; in this and succeeding tables; these are further qualified with plus and minus signs.

It is clear from Table 1 that when the several carbon sources are autoclaved in distilled water and added to autoclaved BBM, certain of them are inhibitory to the growth of certain *Tetracystis* species, as compared with the control in organic medium alone. Ribose, of all the carbon sources used, inhibits most *Tetracystis*

species, especially when autoclaved. At the other extreme is fructose, in which almost the same results were obtained with autoclaved and Seitz-filtered material, neither inhibitory as compared with the BBM control.

On the basis of the data summarized in Table 1, the several species of *Tetracystis* may be divided among 4 groups as follows:

Group 1: Growth not markedly affected by supplementary carbon compounds—*T. pampae, T. aplanosporum, T. intermedium*

Group 2: Growth poor, in inorganic medium alone and with supplementary carbon sources—*T. texensis*

Group 3: Growth affected by supplementary carbon compounds but with poor growth in acetate—*T. aeria* (both C-6 and Pa-3); *T. isobilateralis, T. illinoisensis, T. excentrica, T. pulchra, T. dissociata, T. aggregata.*

Group 4: Growth affected by supplementary carbon compounds; however, growth excellent in acetate—*T. tetrasporum*

Furthermore, inspection of cultures in the sugar- and acetate-supplemented media after 2 months in darkness revealed that the only heterotrophism in *Tetracystis*, under conditions of the present experiment, was exhibited by the 2 isolates (C-6 and Pa-3) of *T. aeria* with both glucose and fructose and by *T. pampae* in acetate. No growth of the other organisms occurred in darkness in the control or in media containing xylose, ribose, arabinose, glucose, fructose, or sodium acetate.

Data on "recovery" in light of the organisms after 2 months in darkness in BBM, with and without the carbon sources, are summarized in Table 2. The patterns of inhibition are similar to those in Table 1, but they are markedly accentuated.

TABLE 2. *Growth of Tetracystis species (recovery) in light at 2 weeks following a 2-month period in darkness*

Type of sterilization	BBM	Xylose		Ribose		Arabinose		Glucose		Fructose		Na-acetate	
	A	B	A	B	A	B	A	B	A	B	A	B	A
T. aeria (C-6)	N	N	N	T	T	T	T	E	E	E	E	N	N
T. aeria (Pa-3)	N	T	N	T	T	T	T	E	E	E	E	N	N
T. isobilateralis	N	E	E	E	E	E	E	E	E	E	G	T	T
T. aggregata	N	G	G	F	T	G	G	T	G	F	T	T	N
T. dissociata	N	T	N	T	N	F	N	F	F	F	F	N	N
T. pampae	N	E	E	E	E	E	E	E	E	E	E	E	E
T. aplanosporum	N	E	E	E	E	E	E	E	E	E	E	T	T
T. intermedium	N	G	F	E	G	E	E	E	E	E	E	T	T
T. tetrasporum	N	F	T	T	N	E	G	F	F	F	T	N	N
T. texensis	N	T	N	N	N	N	N	T	N	N	N	N	N
T. excentrica	N	T	N	N	N	T	N	F	N	F	N	N	N

A = Autoclaved
B = Seitz-filtered

Sensitivity to crystal violet

Mattox and Bold (1962) used tolerance to crystal violet as a criterion in their classification of certain ulotrichacean algae. However, as indicated, the medium must be inoculated immediately after preparation, because the inhibitory efficacy of crystal violet deteriorates markedly with prolonged storage in the light, but less so in darkness (Table 3).

One gram of Difco crystal violet powder was dissolved in 100 ml of distilled water to make a 1.0% stock solution. To liquid BBM was added sufficient stock solution to make the following final concentrations of crystal violet: 1 part crystal violet/100,000 parts BBM (0.001%); 1 part crystal violet/50,000 parts BBM (0.002%); 1 part crystal violet/25,000 parts BBM (0.0045%); and 1 part crystal violet/10,000 parts BBM (0.01%). The crystal violet media were then solidified with Difco Bacto powdered agar (1.6% concentration) and used immediately.

TABLE 3. *Growth of Tetracystis species (at 2 weeks) on BBM agar with various concentrations of crystal violet (Fig. 107)*

	Inoculated immediately after preparation				Inoculated 2 weeks after preparation			
Concentration of cr. violet[a]	1/100	1/50	1/25	1/10	1/100 Lt.	1/100 Dk.	1/50 Lt.	1/50 Dk.[b]
T. aeria (C-6)	N	N	N	N	E	ND	E	ND
T. aeria (Pa-3)	N	N	N	N	ND	ND	ND	ND
T. isobilateralis	T	T	T	N	E	ND	E	T
T. aggregata	T	T	N	N	E	ND	E	ND
T. dissociata	T	T	T	N	E	T	E	T
T. tetrasporum	T	F	T	N	E	F	G	T
T. excentrica	F	F	T	N	N	ND	N	ND
T. intermedium	E	E	E	T	E	E	E	E
T. texensis	G	G	F	T	E	G	N	N
T. pampae	E	E	G	T	E	ND	E	ND
T. aplanosporum	E	E	E	T	E	E	E	E

[a] 1/100 = 1 pt. crystal violet to 100,000 parts BBM.
1/50 = 1 pt. crystal violet to 50,000 parts BBM.
1/25 = 1 pt. crystal violet to 25,000 parts BBM.
1/10 = 1 pt. crystal violet to 10,000 parts BBM.
[b] Storage during 2-week period before inoculation.

The data in Table 3 and Fig. 107 indicate differential sensitivity of the *Tetracystis* isolates to varying concentrations of crystal violet. For example, *T. intermedium*, *T. texensis*, *T. pampae*, and *T. aplanosporum* are clearly less sensitive than other isolates to a concentration of crystal violet of 1: 25000. Other species (*T. aeria*, both strains, *T. isobilateralis*, *T. aggregata*, *T. dissociata* and *T. tetrasporum*) cannot tolerate crystal violet even in as dilute a solution as 1: 100,000!

Extracellular amylasic activity

Extracellular amylasic activity of green algae has been explored as a possible taxonomic criterion by Bischoff and Bold (1963) and the same criterion has been tested in the several *Tetracystis* species.

For testing amylasic activity, starch agar was prepared by using a 0.01% concentration of ACS-grade starch in BBM solidified with 1.6% Difco Bacto powdered agar. The presence of starch was indicated by I_2KI solution which was made by dissolving 10.0 g of KI in 500 ml distilled water and subsequently adding 2.0 g of metallic iodine.

The results of duplicate tests run twice are presented in Table 4. The degree of activity was appraised by the width of the clear (starch-digested) zone which was evoked by adding I_2KI. From the data in Table 4 and Fig. 108, it is apparent that the species of *Tetracystis* differ consistently in their extracellular amylasic activity.

TABLE 4. *Amylasic activity of Tetracystis species after growth on BBM supplemented with 0.01% starch*[a] *and grown for 2 weeks under standard conditions (Fig. 108)*

Organism	Amylasic activity
T. excentrica	slight
T. texensis	slight
T. intermedium	slight
T. aplanosporum	fair
T. aeria (C-6)	good
T. aeria (Pa-3)	good
T. dissociata	good
T. isobilateralis	good
T. aggregata	good
T. pampae	good
T. tetrasporum	excellent

[a] Potato.

Growth in several complex media

Growth in certain complex media, while useful in routine detection of contamination, sometimes has also proven to be consistently differential. In the present investigation, differential growth of the *Tetracystis* species was exhibited after 3 weeks in Difco Nutrient Broth, Difco Nutrient Agar, 1% yeast agar,[1] and Difco Thioglycollate medium (Table 5).

[1] 5 g Difco yeast extract dissolved in 1 liter of de-ionized water and solidified with 16 g of agar.

TABLE 5. *Growth of Tetracystis species on various complex media*[a]

Organism	Growth on nutrient agar	Growth on yeast agar	Growth in nutrient broth	Growth in thioglycollate medium
T. aeria (C-6)	G	G	G	F
T. aeria (Pa-3)	G	G	G	F
T. isobilateralis	N	G	N	G
T. dissociata	N	G	N	F
T. aggregata	N	G	N	G
T. excentrica	G	N	N	F
T. texensis	G	N	N	G
T. aplanosporum	G	G	N	G
T. intermedium	G	G	F	G
T. tetrasporum	G	G	F	G
T. pampae	G	G	G	G

[a] At 3 weeks incubation.

Sensitivity to antibiotics

The use of antibiotics for differentiation of algal isolates has been a practice in this laboratory for some years (Deason and Bold, 1960; Chantanachat and Bold, 1962; Mattox and Bold, 1962; Bold and Parker, 1962; Bischoff and Bold, 1963). From more than 30 antibiotic agents which were screened, 9 were chosen for further use.

Selective inhibitory action of certain antibiotics was demonstrated by preparing Petri dishes of BBM Ion Agar[1] (1.0%) to which an inoculum was added before the medium had sodidified. After gelation, paper tabs impregnated with various antibiotics[2] were aseptically dispensed onto the surface of the agar. The plates were then inverted and stored under standard conditions, for 2 weeks, after which they were examined and the degree of inhibition of growth, if any, was recorded (as measured by a clear zone circumscribing the antibiotic disc.) The data are presented in Table 6.

One isolate of *T. aeria* (P_a-3) was not sensitive to any of the antibiotics used, while the other strain of the same species was sensitive only to Polymyxin B. Furthermore, no two of the isolates tested had identical responses of sensitivity, so that this is a helpful supplement in classifying the organisms.

The writers have carefully considered data obtained in each of the tests summarized in Tables 1–6[3] and have constructed species groupings based on responses of the algae to a given procedure. These species groupings are summarized herewith.

[1] Oxoid Ion agar No. 2, Consolidated Laboratories, Inc., Chicago Heights, Illinois.
[2] Sensi Disc Method, Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.
[3] And other data.

TABLE 6. *Response of Tetracystis species to certain antibiotic agents (Sensi-disc method)*

	CL[a]	FX	SM	N	PB	E	SD	G	DS
T. aeria (C-6)	—	—	—	—	+[b]	—[c]	—	—	—
T. aeria (Pa-3)	—	—	—	—	—	—	—	—	—
T. isobilateralis	+	+	—	+	+	+	—	—	Sl[d]
T. aggregata	+	+	—	+	+	—	—	—	+
T. dissociata	—	+	—	+	+	—	+	—	+
T. intermedium	—	+	—	+	+	—	—	—	+
T. texensis	+	+	—	+	+	—	—	ND[e]	—
T. excentrica	+	+	—	+	+	—	+	ND	+
T. pampae	+	+	—	+	+	+	+	—	+
T. aplanosporum	—	+	—	Sl	+	—	—	—	ND
T. tetrasporum	+	+	+	+	+	+	—	+	ND

[a] Antibiotics used and their concentrations:
CL = Coly-Mycin, 10.0 mcg.
FX 100 = Furoxone, 100.0 mcg.
SM 1 = Sulfamerazine, 1.0 mg.
N 30 = Neomycin, 30.0 mcg.
PB 300 = Polymyxin B, 300.0 units.
E 15 = Erythromycin, 15.0 mcg.
SD 1 = Sulfadiazine, 1.0 mg.
G 2 = Gantrisin, 2.0 mg.
DS 10 = Dihydrostreptomyocin, 10.0 mcg.
[b] + indicates a definite inhibition.
[c] — indicates no inhibition.
[d] Sl indicates a slight inhibition.
[e] ND = no data (disc fell off agar).

A. From Table 1. Growth on various carbon sources in the light

Group 1:
T. pampae
T. aplanosporum
T. intermedium

Group 2:
T. texensis

Group 3:
T. aeria (both C-6 and P_a-3)
T. isobilateralis

Group 3 (continued):
T. illinoisensis
T. excentrica
T. pulchra
T. dissociata
T. aggregata

Group 4:
T. tetrasporum

B. From Table 2. Growth on various carbon sources in the light following a prolonged dark period (recovery)

Group 1:

T. aeria (both C-6 and P_a-3)

Group 2:

T. aplanosporum

Group 3:

T. pampae

Group 4:

T. dissociata
T. texensis
T. excentrica
T. tetrasporum

Group 5:

T. aggregata

Group 6:

T. intermedium

C. From Table 3. Growth in BBM supplemented with crystal violet

Group 1:

T. aeria (both C-6 and P_a-3)

Group 2:

T. isobilateralis
T. aggregata
T. dissociata
T. tetrasporum
T. excentrica

Group 3:

T. intermedium
T. pampae
T. aplanosporum

Group 4:

T. texensis

D. From Table 4. Amalysic activity

Group 1:

T. tetrasporum

Group 2:

T. aeria (both C-6 and P_a-3)
T. dissociata
T. isobilateralis
T. aggregata
T. pampae

Group 3:

T. aplanosporum

Group 4:

T. excentrica
T. texensis
T. intermedium

E. From Table 5. Growth in Thioglycollate Broth

Group 1:
- *T. aeria* (both C-6 and P_a-3)
- *T. dissociata*
- *T. excentrica*

Group 2:
- *T. isobilateralis*
- *T. aggregata*

Group 2 (continued):
- *T. pampae*
- *T. texensis*
- *T. tetrasporum*
- *T. intermedium*
- *T. aplanosporum*

Growth in Nutrient Broth (Table 5)

Group 1:
- *T. aeria* (both C-6 and P_a-3)
- *T. pampae*

Group 2:
- *T. tetrasporum*
- *T. intermedium*

Group 3:
- *T. isobilateralis*
- *T. aggregata*
- *T. dissociata*
- *T. excentrica*
- *T. texensis*
- *T. aplanosporum*

Growth on Nutrient Agar and Yeast Agar (Table 5)

Group 1:
- *T. aeria* (both C-6 and P_a-3)
- *T. aplanosporum*
- *T. intermedium*
- *T. tetrasporum*
- *T. pampae*

Group 2:
- *T. excentrica*
- *T. texensis*

Group 3:
- *T. isobilateralis*
- *T. dissociata*
- *T. aggregata*

F. From Table 6. Response to certain antibiotics

Group 1:
- *T. aeria* (both C-6 and P_a-3)

Group 2:
- *T. tetrasporum*

Group 3:
- *T. isobilateralis*
- *T. aggregata*
- *T. pampae*
- *T. excentrica*
- *T. texensis*

Group 4:
- *T. aplanosporum*
- *T. dissociata*
- *T. intermedium*

G. Facultative heterotrophy

Group 1:
T. aeria (both C-6 and P_a-3)

Group 2:
T. aplanosporum

Group 3:
T. tetrasporum

Group 4:
T. pampae

Group 5:
T. aggregata
T. isobilateralis
T. dissociata
T. intermedium
T. texensis
T. excentrica

H. Growth on BBM supplemented with vitamins

Group 1:
T. aeria (both C-6 and P_a-3)
T. pulchra

Group 2:
T. texensis
T. dissociata

Group 3:
T. pampae
T. excentrica
T. aplanosporum
T. intermedium

Group 4:
T. isobilateralis
T. illinoisensis
T. aggregata

Group 5:
T. tetrasporum

From these data, it is clear that no single species has a unique pattern of response to *all* of the tests; however, certain species seem to fall into groups which indicate significant relationships. So that the reader may visualize the over-all picture more conveniently, Table 7 has been constructed to correlate the number of different physiological tests (out of a total of 10[1]) in which a given species (left hand column) appears in a group by itself, or with 1 additional species, 2 additional species, etc., up to the case of 6 additional species. For example, both isolates of *T. aeria* appear as the only species comprising the given group in 4 different physiological tests. In only 1 physiological test, however, *T. aeria* does appear in a group consisting of 6 additional species, etc.

The most distinctive species on the basis of this analysis of physiological attributes are: (1) *T. aeria* which stands alone in 4 different physiological tests; and (2) *T. tetrasporum* which is unique in its response to 5 of the 10 experimental procedures.

[1] An experiment adding vitamin supplement to the basal medium, did not prove very differential. This, while not discussed in the text, is included in Table 7.

TABLE 7. *Summary of the number of physiological tests in which a given species is unique or similar to other species*

Species	O[a]	1	2	3	4	5	6	7
T. aeria (C-6) and (Pa-3)	1111[b]	11	1	....	11	....	1	3
T. isobilateralis	....	1	11	....	111	11	11	7
T. aggregata	1	....	11	....	111	111	1	7
T. dissociata	....	1	111	1	11	11	1	5
T. excentrica	....	11	1	11	11	11	1	5
T. texensis	11	11	1	1	1	11	1	4
T. intermedium	1	1	1111	1	1	1	1	3
T. pampae	11	1	11	1	111	....	1	4
T. aplanosporum	11	1	111	1	1	1	1	3
T. tetrasporum	11111	1	....	1	11	....	1	3

[a] The number of additional species sharing a given physiological response with the species in the left-hand column.

[b] The number of different physiological tests (out of a total of 10) in which a given species (left-hand column) appears in a grouping by itself or with a number of other species shown across the top of the table.

Tetracystis isobilateralis, *T. aggregata*, *T. dissociata*, and *T. excentrica* may be considered in a physiological species grouping in that they all form groups with at least 4 additional algae in over half of the 10 physiological tests. (See Roman numerals in column 7, Table 7.)

Likewise, *T. texensis*, *T. intermedium*, *T. pampae*, and *T. aplanosporum* may be considered a physiological species grouping in that they all form groups with at least 3–4 additional algae in over half of the 10 physiological tests. (See column 7, Table 7.)

Finally, it is quite possible to identify and segregate 7 of the isolates of *Tetracystis* tested on the basis of their pattern of physiological response alone. Thus, a diversity of physiological tests can provide useful data for supplementing the morphological criteria used in classifying *Tetracystis* and other algal species. This will be borne out by the correlations to be made later in this study on the bases of ultrastructural and immunochemical evidence.

III. Electron Microscopy of *Tetracystis* and Certain *Chlorococcum* Species

In addition to light-microscopic studies of species of the genera *Tetracystis* and *Chlorococcum*, it became clear upon preliminary examination with the electron microscope that ultrastructural differences within and between these genera would provide additional, precise, continuously available criteria of taxonomic signifi-

cance. Furthermore, the exact nature of cell division, especially in *Chlorococcum* and *Tetracystis* was incompletely known through available light-microscopic studies (Herndon, 1958; Deason and Bold, 1960), and for this reason it seemed worthwhile to investigate with the electron microscope[1] the processes of cytokinesis leading to the formation of both daughter vegetative cells and zoospores, in *Tetracystis*, and to zoospores alone, in *Chlorococcum*. *Tetracystis* provided excellent material for studies of pyrenoid organization and formation. Accordingly, a special section has been devoted to this topic.

A. MATERIALS AND METHODS

Unialgal and bacteria-free cultures of 11 species of *Tetracystis* and of 4 species of *Chlorococcum* were selected for electron-microscopic investigations (Table 8).

TABLE 8. *Algae investigated electron microscopically*

Tetracystis isobilateralis	*Tetracystis pampae*
T. aggregata	*T. illinoisensis*
T. aeria (C-6) (Pa-3)	*T. pulchra*
T. excentrica	*Chlorococcum perforatum*
T. dissociata	*C. echinozygotum*
T. texensis	*C. multinucleatum*
T. intermedium	*C. sp.* (tetra isolate)
T. aplanosporum	

All the organisms were maintained on Bold's Basal Medium (BBM) solidified with 1.6% agar. When culturing the algae in preparation for electron-microscopic studies, 10 ml of liquid medium were added aseptically to each agar slant, and the latter was then immersed for about 5 sec in an ultrasonic water bath. Six drops of the resultant homogeneous cell suspension were transferred aseptically by sterile Pasteur pipettes to 15 × 100 mm Petri dishes of BBM agar. The latter were then vigorously swirled to disperse the suspension evenly over the agar surface. The Petri dish cultures were maintained upside-down under standard conditions of culture for from 3 days to 3 weeks, depending on the time required to obtain the desired life-cycle stage for study. Growth in late log phase (ca. 12 days after inoculation) under these conditions provided adequate material with most of the desired life cycle stages present; oils, starches, and other reserve metabolites were minimal at this phase.

The algal material was harvested by scraping from the agar surface with the edge of a microscope slide and transferring the mass to a spatula for immediate immersion in the desired fixative.

[1] An R.C.A. E.M.U. 3 D Electron Microscope operated at 50 kv was used for the examination of all material throughout this study.

Various fixation procedures were used in order to determine the fixative best suited for demonstration of the several cellular organelles. In terms of penetration, most of the algae studied presented no problems. Occasionally, however, when some of the *Tetracystis* species had entered the stationary phase of growth and undergone wall thickening, the fixatives did not penetrate well.

A preliminary study was made with 6 different, freshly prepared permanganate fixatives, namely, Ca^{++}, Sr^{++}, Mg^{++}, Na^{+}, Li^{+}, and K^{+} permanganates (Fig. 109–114). All permanganates were prepared as a 2% aqueous solution in deionized water. In addition, K^{+} and Li^{+} permanganates were prepared and tested at 4% concentration. The schedule for fixation, dyhydration, and embedding of material treated with these 6 permanganate fixatives is shown in Table 9. The images obtained with these fixatives in the test organism, *Tetracystis isobilateralis*, are shown in Fig. 109–114 and will be discussed below.

Later in this study, the effects of glutaraldehyde fixation on material to be treated with OsO_4 and $LiMnO_4$ were investigated. The fixation and post-fixation schedule for this test series, using *Tetracystis isobilateralis*, is shown in Table 10.

A given volume of 5% glutaraldehyde (diluted from a 25% stock in which the

TABLE 9. *Fixation, dehydration, and embedding schedule of test series with 6 different permanganate fixatives, using Tetracystis isobilateralis (A6–2–3)*

Treatment	Methods	
1. Fixation	a. Six different permanganates at 2%and 4% (unbuffered, aqueous).	
	b. Fixation time: 70 min (5° C)	
2. Rinse	a. Three times with distilled H_2O (5° C)	
3. Dehydration	25% EtOH	10 min (5° C)
	50% EtOH	10 min (5° C)
	75% EtOH	20 min (R. T.)[a]
	95% EtOH	10 min (R. T.)
	100% EtOH	10 min (R. T.)
	100% EtOH	10 min (R. T.)
4. Infiltration	Propylene oxide	15 min (R. T.)
	Propylene oxide	30 min (R. T.)
5. Embedding	25% Plastic/75% Propylene oxide	1 hr (R. T.)
	50% Plastic/50% Propylene oxide	1 hr (R. T.)
	75% Plastic/25% Propylene oxide	21 hr (R. T.)
	100% Plastic	24 hr (R. T.)
6. Polymerization	100% Plastic	24 hr (110° C)

[a] Room temperature.

TABLE 10. *Glutaraldehyde fixation, permanganate and osmium post-fixation, and post-treatments schedule with Tetracystis isobilateralis*

Treatment		Methods		Temp. time
1. Fixation (for both $LiMnO_4$ and Osmium, OsO_4, fixed materials)		2.5% Glutaraldehyde buffered to pH 7.4 with equal volume of Cacodylate[a]	5° C	16 days
2. Rinse	A. Material to be treated in $LiMnO_4$	a. Two changes of Cacodylate[b] followed by:	5° C	10 min each
		b. Two changes deionized H_2O		
	B. Material to be treated in OsO_4	a. Four changes in Cacodylate[b]	5° C	10 min each
3. Post-fixation	A. $LiMnO_4$	a. 2% aqueous	5° C	2 hr
	B. OsO_4	b. 1%, buffered to pH 7.4 with Cacodylate[a]	5° C	2 hr
4. Post-treatments	A. For $LiMnO_4$	a. Four changes of deionized	5° C	5 min each
	B. For OsO_4	b. Four changes in Cacodylate[b]	5° C	5 min each

[a] Cacodylate—50 ml of 0.2 M sodium cacodylate plus 2.7 ml 0.2 M HCl diluted to a total of 200 ml with deionized H_2O.

[b] Cacodylate—0.2 M sodium cacodylate only.

pH must be no higher than 4) was mixed immediately before use with an equal volume of Cacodylate buffer (pH = 7.4). The results of the glutaraldehyde fixation series are illustrated photographically in Fig. 115–120.

During the first phase of this work, propylene oxide was used as the intermediate between the alcohol and plastic series. This procedure worked very well, but because of the expense of the chemical and the possible health hazards involved, 99.9% purified acetone was utilized in place of propylene oxide and worked equally well. The plastic was diluted also with acetone for the embedding procedure.

The following formulation of plastic mixture #1, as commonly employed at the Electron Microscope Laboratory at The University of Texas, was used throughout this study:

Plastic Mixture #1[1]
15 ml Araldite "M"
25 ml Epon 812
55 ml DDSA
2–4 ml dibutyl phthlate

[1] H. H. Mollenhauer (1964).

To use: add 1 drop of DMP-30 for each milliliter of 100% plastic as an accelerator for hardening (this includes the diluted plastics at the initiation of the embedding process).

Plastic mixture #1 may be prepared separately in 2 parts: Part 1—Araldite "M," Epon 812, and dibutyl phthlate; Part 2—DDSA. Combine separate parts immediately before use in the ratio of 9 parts of (1) and 11 parts of (2). This procedure avoids the formation of a very viscous plastic mixture which develops in 1–2 weeks if both parts are mixed during initial preparation.

Embedding was begun by introducing the material into a 25% plastic mixture diluted with propylene oxide or 99.9% acetone. The material was left in this mixture for 1 hr, then was transferred to a 50% plastic mixture for 1 hour and then to a 75% plastic mixture for 21 hr (Table 9). In some schedules, the algal material was left in the 75% plastic mixture for 3 days without harmful effects. Following the 75% dilution, the material was transferred to 100% plastic and immediately afterwards was poured into molds (aluminum cups or "boats," or plastic snap-on bottle caps; the plastic caps were re-usable, and no mold-release was needed when these were used). The material was kept in the molds (which were placed in 100-ml glass Petri dishes to keep out dust and contamination) at room temperature for at least 12 hr before being placed in an oven at 110° C. The plastic mixture polymerized into a sufficiently hard block after 12–16 hr at oven temperature.

Embedding schedules using a dilution series of 33%, 66%, and 100% plastic worked equally well, especially when freshly prepared plastics were used.

All sectioning was carried out with a diamond knife, Type C (from Ge-Fe-Re: Via Marillima, Frosinone, Italia) on a Porter Blum Microtome. Cut sections were placed on 300-mesh grids which had been hand-polished on 1 side and then dipped in a solution of 5% Formvar in ethylene dichloride. No cleaning of the grids was necessary as long as they were adequately coated with Formvar. Coating of the grids when used in conjunction with plastic mixture #1 was also unnecessary.

Most permanganate-fixed materials prepared in this study were routinely post-stained after sectioning in Millonig's PbOH (Millonig, 1961) for 5 min. A 5% $Ba(OH)_2$ solution was employed on occasion as a post-stain to resolve wall structure, Golgi vacuolar products, and metabolic products such as starch, oils, etc.

Material fixed in the glutaraldehyde series was post-stained in several ways. The OsO_4-fixed material was post-stained in a saturated solution of Uranyl acetate for 15 min (Karnowvsky, 1961) followed by Reynolds' lead citrate post-stain (5 min) (Reynolds, 1963). $LiMnO_4$-fixed material was post-stained for 5 min only in the Reynolds' post-stain.

B. RESULTS

1. Fixation images

Two categories of permanganate-fixation images resulted from the permanganate series with *Tetracystis isobilateralis*. Magnesium, calcium, and stronium per-

manganates yielded the first type, notably that with swollen chloroplast lamellae (Fig. 112–114). Within this series $Sr(MnO_4)_2$ resulted in a granular configuration of the pyrenoid matrix. The latter was preserved differently from the chloroplast matrix, and a fibrillar appearance was often present (Fig. 114–2).[1] The cytoplasm and its lamellar components appeared essentially similar with calcium, magnesium, and stronium permanganates.

The second category of fixation image was obtained with sodium, lithium, and potassium permangantes (Fig. 109–111). Here, there was much less distortion and swelling of the chloroplast lamellar components. The fixation image was most granular with potassium and sodium permanganates (Fig. 110, 111). The best fixation image within this test series was obtained with 2% $LiMnO_4$ (Fig. 109). For this reason the latter was used for the majority of species subsequently studied.

Later in this investigation the writers' attention was called to a glutaraldehyde fixation technique (Sabatini, Bensch, and Barrnett, 1962; Ledbetter and Porter, 1963). The authors' modifications of these techniques for the algal material under investigation are presented in Table 10. The results of this study are presented in Fig. 115–120. In glutaraldehyde fixation, followed by OsO_4 or $LiMnO_4$ treatment, the preservation was superior to that of material in OsO_4 or $LiMnO_4$ without prior glutaraldehyde treatment.

Lithium permanganate-treated material, first fixed with glutaraldehyde, is similar in many aspects to osmium-fixed material. It is of interest that ribosome-like particles (Fig. 119–6), "osmiophilic particles" (Fig. 116–4), and nucleolar material (Fig. 116–3) are observable in permanganate-treated preparations fixed with glutaraldehyde. The above mentioned cellular components are not preserved in algal material fixed directly in $LiMnO_4$ (Fig. 127–131).

Thus, permanganate fixation may be made to approach more closely that of osmium when the former is used in conjunction with glutaraldehyde. This schedule maintains the well-known advantages of permanganate fixation of plant material with respect to preservation of membrane and lamellar systems.

Osmium-treated material of *Tetracystis isobilateralis* also is preserved much better when fixed with glutaraldehyde. In the writers' algal material, when 1% OsO_4 was employed without glutaraldehyde fixation, the lamellar systems, particularly those of the chloroplast, swelled markedly, as they do with calcium-, magnesium-, and stronium-permanganate fixation. Such a swelling effect is greatly reduced when glutaraldehyde is employed first (Fig. 115, 117, 118). However, such material is of very low contrast and is only slightly improved with Reynolds' and Uranyl acetate post-staining procedures.

Thus, $LiMnO_4$ treatment provided the best fixation image of all when prior fixation in glutaraldehyde was instituted. Such a fixation image may indicate a

[1] The number after the dash in each case refers to the numbered arrow in the figure.

heretofore unrealized potential with permanganate preservation, particularly with reference to algal material.

2. *Comparative study of cellular organelles in* Tetracystis

(1) The chloroplast

The ultrastructure of the chloroplast in all species of *Tetracystis* is essentially similar to that of other chlorophycean algae (Gibbs, 1962c; Lang, 1963) in that the plastids are limited by a double membrane which surrounds a granular stroma (Fig. 125). The latter contains a number of lamellar structures. The unit structures, which are flattened sacs, are termed the discs (Fig. 136–3). Varying amounts of starch are present within the chloroplast stroma (Fig. 132) and are also associated with the pyrenoid (Fig. 143).

Variations in gross chloroplast form have been used as taxonomic attributes at the generic level in the Chlorococcales and Chlorosphaerales (Starr, 1955; Herndon, 1958). At the specific level, such chloroplast attributes as presence or absence of perforations, fissuring, mass, and pyrenoid number and position have been found reliable in delineating species. In the present investigation, attention has been devoted to these same attributes electron microscopically. Light-microscopic study has revealed that the several species of *Tetracystis* may be divided into 3 categories on the basis of chloroplast mass (thin, thick, and intermediate). In this connection, the electron-microscopic data support those obtained by light microscopy. These categories and their attributes along with the type of fissuring, if present, are summarized in Table 11.

Ultrastructurally, the organization of the chloroplast has been found reliable in segregating species of *Tetracystis* on the basis of lamellae. In 1 species, *Tetracystis pampae* (Fig. 156–158), the discs are always associated in three's. In the remaining species, both number and pattern of disc association may vary, or the number alone may vary and the pattern of associated discs may be distinct (Table 12).

In the latter case, 3 basic patterns of disc association have been observed, and there is some evidence of intergradation. In the first pattern, irregularly alternating groups of 1–2 discs occur with groups of 3–5 (Fig. 155–6). In the second, there are irregularly dispersed groups of greater variability with respect to number of discs associated, these numbers ranging from 2–10 (Fig. 133). The third pattern of disc association is composed of 2 to 6–8 discs per group (Fig. 121).

The second and third patterns of disc association may be subdivided further on the basis of presence or absence of reticulations between the groups of associated discs. These reticulations consist of end-to-end association of curving, single lamellae, usually near the pyrenoid (Fig. 121–2). Finally, certain *Tetracystis* species have both variable patterns and numbers of associated discs (Table 12, III).

Other investigators apparently have not reported ultrastructural differences in the chloroplast useful for taxonomic purposes at the species level. Gibbs (1962c) found that association of chloroplast discs in the Chlorophyta varied in number

TABLE 11. *Chloroplast organization in Tetracystis as revealed by electron microscopy*

Species	Chloroplast mass[a] Thin[b]	Thick[c]	Intermediate[d]	Fissuring Present	Absent	Type of fissuring
T. pulchra (Fig. 152)	+	—	—	—	+	—
T. intermedium (Fig. 144–3)	+	—	—	—	+	—
T. texensis (Fig. 147–1)	+	—	—	—	+	—
T. excentrica (Fig. 143–7)	+	—	—	—	+	—
T. pampae (Fig. 158–4)	+	—	+	—	+	—
T. illinoisensis (Fig. 141)	+	—	—	+	—	Deep, broad invagination, external only
T. aplanosporum (Fig. 153, 155–7)	+	—	—	+	—	Deep, narrow fissures, external only
T. aeria (C-6) (Fig. 121–3)	—	—	+	+	—	Deep, broad invagination, external only
T. aeria (Pa-3) (Fig. 124–3)	—	—	+	+	—	Deep, broad invagination, external only
T. isobilateralis (Fig. 130–2, -3)	—	+	—	+	—	Both internal and external fissures
T. aggregata (Fig. 134–7)	—	+	—	+	—	Deep, broad invagination, external only
T. dissociata (Fig. 135, 137)	—	+	—	+	—	Both internal and external fissures

[a] As seen in optical section.

[b] Thin: chloroplast mostly thin and parietal, slightly thickened in region of pyrenoid (Fig. 144).

[c] Thick: chloroplast massive, thick, almost filling the cell lumen except for nucleus (Fig. 130).

[d] Intermediate: intermediate between [b] and [c].

from 2 to 6. That she noted such small variation may be explained by her investigation of only a single species of each of 3 genera. Lang (1963) reported the most common number of disc associations to be 3 in members of the Volvocaceae and Astrephomenaceae in which she studied 8 different genera and at least 14 different species. She did not state maximum numbers of associated discs but did indicate that it varied from 3 to many in the same chloroplast. Thus, it appears that internal chloroplast diversity does not always accompany species and generic differences within certain families. On the other hand, the investigations of Ueda (1961) indicate a wide range of variation of chloroplast organization within the Chlorophyta. According to Ueda, disc association varied from 2 to 20 in *Chlamydomonas, Oedogonium,* and *Tetraspora;* from 2 to 40 in *Chlorococcum* sp.; and from 2 to 80 in *Palmella.*

The stability of certain internal chloroplast features may account for consistent differences reported in different species. The consistency of numbers of disc com-

TABLE 12. *Internal chloroplast organization of Tetracystis*

I. Discs consistently associated in 3's.
Tetracystis pampae (Fig. 158–6)
II. Distinct patterns, but variable numbers of associated discs.
A. Groups of 1–2 discs irregularly alternating with, and widely spaced from, groups of 3–5 discs.
Tetracystis aplanosporum (Fig. 155–6)
B. Two-10 associated discs: no definite reticulations between disc systems.
Tetracystis isobilateralis (Fig. 128–2)
T. aggregata (Fig. 133–5)
T. dissociata (Fig. 136–3)
C. Two-6 or 8 associated discs; definite reticulations present.
Tetracystis aeria (C-6) (Fig. 123–6)
T. aeria (Pa-3) (Fig. 125–4)
T. illinoisensis (Fig. 142–5)
III. Pattern and number of associated discs variable.
Tetracystis intermedium (Fig. 145–5)
T. pulchra (Fig. 151–1)
T. excentrica (Fig. 143–7)
T. texensis (Fig. 149–4)

ponents in an association, as observed in *Tetracystis,* is demonstrated also in the discs or tubules which enter the pyrenoid. For example, the pyrenoid of *T. pampae* is traversed by triple discs (Fig. 158–5). In other species in which the chloroplast disc system is more variable, as in *Tetracystis texensis* or *T. isobilateralis,* the disc associations are reduced to 2 or to 1 within the pyrenoid (Fig. 159, 163). When definite patterns of association occur (Table 12), a greater variability is noted among the *Tetracystis* species.

Not only gross, but also ultrastructural, features of chloroplast organization, therefore, are of value in distinguishing species of *Tetracystis.*

(2) The pyrenoid

The pyrenoid is a region of the chloroplast consisting of a homogeneously granular matrix surrounded by starch plates. Lamellar structures may or may not penetrate the pyrenoid matrix.

As far as the writers are aware, there have been no comparative studies of pyrenoid ultrastructure at the species level. Various investigators have described the ultrastructure of the pyrenoid (Albertson and Leyon, 1954; Butterfass, 1957; Chordad and Roullar, 1957; Sager and Palade, 1957; Hovasse and Joyon, 1957; Brody and Vatter, 1959; Gibbs, 1960, 1962b; Ueda, 1961; Lang, 1963; and Murakami, Morimura, and Takamiya, 1963). These studies have been principally

descriptive but include incidental experiments with reference to the occurrence of pyrenoids, their ultrastructure, and the response of pyrenoid ultrastructure to environmental changes.

The pyrenoids of 11 species of *Tetracystis*[1] accordingly were studied with the electron microscope. Several types of pyrenoids could be differentiated at the ultrastructural level. Criteria include lamellar position, number of lamellar structures which penetrate the pyrenoid, their interconnecting patterns, the number and position of pyrenoid starch grains, and pyrenoid shape and size.

The lamellar structures associated with the pyrenoid provide useful taxonomic criteria at the species level first with respect to their location within or upon the pyrenoid matrix (Table 13). Two alternative arrangements have been observed, namely, pyrenoids with largely "peripheral" and those with "internal" lamellae. Peripheral lamellae lie between the periphery of the pyrenoid matrix and the starch grains (Fig. 163). These are limited, single-disc systems at 1 or more loci along the surface of the pyrenoid matrix (Fig. 163–3,–4). The lamellae show a higher degree of undulation where they are single.

TABLE 13. *Pyrenoid organization among the presently known species of Tetracystis*[1]

I. Pyrenoid enclosed by a single starch grain.
   Chlorococcum sp. (tetra)

II. Pyrenoid surrounded by 2 starch grains.
   Tetracystis intermedium
   T. excentrica
   T. texensis
   T. pulchra

III. Pyrenoid surrounded by many starch grains.
   A. Single-disc (tubular) system
      T. aplanosporum
   B. Triple-disc system
      T. pampae
   C. Double-disc system
      a. Disc pattern contorted, pyrenoid less than 5 μ diameter.
         T. aeria (C-6)
         T. aeria (Pa-3)
         T. dissociata
         T. illinoisensis
      b. Disc pattern largely parallel and straight, pyrenoid more than 5 μ diameter.
         T. isobilateralis
         T. aggregata

[1] And of 1 species of *Chlorococcum*.

Species with internal pyrenoid lamellae could be subdivided on the basis of number of associated lamellar discs penetrating the pyrenoid. These lamellar discs were single (Fig. 165), double (Fig. 159, 160), or triple (Fig. 161). Regardless of the condition of the culture and of the fixation method, the number of associated discs within the pyrenoid of a given species of *Tetracystis* is constant. When single elements penetrate the pyrenoid, the individual components may be tubular (Fig. 164–5) or flattened cylinders (Fig. 164–6). In *Tetracystis aplanosporum,* these tubules or flattened cylinders branch and occasionally (and only locally) cohere to form areas of appressed double or triple aggregates. That these lamellar elements penetrating the pyrenoid of *T. aplanosporum* are flattened cylinders or tubules can best be illustrated by comparison with the lamellae within the pyrenoid of *Chlorococcum multinucleatum* in which a tubular, reticulate, network of lamellar components is found (Fig. 166–8,–9).

In pyrenoids containing paired lamellae, the latter generally occur as broad sheets or plates which contain pores (Fig. 162–4). The double-disc systems were either contorted in some species (Fig. 160) or largely straight and parallel in others (Fig. 159).

Only 1 species of *Tetracystis,* namely, *T. pampae,* has a triple-disc system of lamellae penetrating the pyrenoid. Such a system is merely a continuation of the triple-disc lamellar system of the chloroplast itself (Fig. 161–3). That this triple-disc system in both pyrenoid and chloroplast is so far unique, not only within the genus *Tetracystis,* but also among the Chlorophyta, is supported by Gibbs' (1962b) statement that "No green algae have been observed yet in which the pyrenoid matrix is traversed by bands containing as many discs as do the bands in the chloroplast proper."

The number and position of starch grains about the pyrenoid are consistent and very useful comparative criteria. There may be 1 starch grain (Fig. 165), 2 starch grains (Fig. 163), or many starch grains (Fig. 159, 160) about the pyrenoid. Two starch grains are inevitably associated with ellipsoidal pyrenoids within the genus *Tetracystis* (Fig. 163–2). Furthermore, ellipsoidal pyrenoids always have lamellae restricted to the periphery of the matrix, and they are always reduced to a single-disc system at one or more points.

Pyrenoids with many starch plates are never ellipsoidal but may be spherical or irregular (Fig. 121, 132). Observations of spherical pyrenoids with light microscopy show little of taxonomic value except for their size and position; however, observations with the electron microscope reveal significant taxonomic differences with respect to internal pyrenoid structure not observable in light microscopy.

The species of *Tetracystis* may be grouped into 5 categories on the basis of pyrenoid organization. This diversity of pyrenoid structure is greater in *Tetracystis* than among all 7 genera of the Chlorophyta studied by Gibbs (1962b). The gross structure of the pyrenoid and the variation of the lamellar structures which pene-

trate or associate with it appear to be very reliable and useful supplementary data in elucidating the taxonomy of *Tetracystis*.

(3) The mitochondrion

It has been well established that mitochondria occur in the cells of green algae (Sager, 1959; Lang, 1963, etc.). Comparative study of the 11 species of *Tetracystis* has revealed considerable variation in the mitochondria, especially with respect to their size and form.

The mitochondria of the several species range from small, bacilliform organelles 0.1 μ in length (Fig. 121–4) to larger, branched or unbranched structures up to 8 μ in length (Fig. 131–5). Furthermore, the mitochondria differ in form. Two basic types have been recognized: (1) cylindrical mitochondria (Fig. 147–2, small; and Fig. 141–2, large), straight or curved (even vermiform) (Fig. 156–2); and (2) compressed, ribbon-like mitochondria, unbranched or branched (Fig. 133–4, 131–5. These 2 categories are mutually exclusive; that is, species of *Tetracystis* with compressed mitochondria seem never to have cylindrical ones.

Table 14 summarizes the data obtained with respect to mitochondrial organization in the genus *Tetracystis*. As is clear from the table, *Tetracystis isobilateralis* can be distinguished at once on the basis of electron microscopy from all other known species by its compressed, branched, giant mitochondria (Fig. 130, 131). These mitochondria seem to be larger than any so far reported in the algae.

It is clear also from Table 14 that small, simple mitochondria characterize a

TABLE 14. *Types of mitochondria in the genus Tetracystis*

I. Cylindrical
Tetracystis aeria (C-6) (Fig. 121–4)
T. aeria (Pa-3) (Fig. 126–5)
T. excentrica (Fig. 143–8)
T. texensis (Fig. 147–2)
T. aplanosporum (Fig. 153–3)
T. intermedium (Fig. 144–1)
T. pampae (Fig. 156–2)
T. pulchra (Fig. 152–5)
T. illinoisensis (Fig. 141–2)
II. Compressed
A. Rarely branched
T. aggregata (Fig. 133–4)
T. dissociata (Fig. 137)
B. Frequently branched
T. isobilateralis (Fig. 131–5)

majority of the species of *Tetracystis*. No significant differences among the cristae of the several types of mitochondria were observed.

(4) The Golgi apparatus

Although a number of investigators have reported the presence of Golgi bodies in the cells of green algae (Sager, 1959; Lang, 1963, among others), no special intensive study of these organelles seems to have been undertaken. The availability of material of *Tetracystis* with these organelles especially well preserved impelled the writers to study them carefully and comparatively.

These comparative studies of 11 species of *Tetracystis* have revealed that 2 basic types of Golgi organization are present, namely, that in which the groups of cisternae are not distinctly associated with an extension of the nuclear envelope (Fig. 131–4) and that in which they are so associated (Fig. 143–9). In the latter case, extensions of the outer nuclear membrane protrude and the protrusion always encompasses the Golgi apparatus. This type of relationship was first observed by Moner and Chapman (1960) in *Pediastrum,* and the nuclear protrusion was designated an "amplexus" by Lang (1963).

The Golgi apparatus itself may be differentiated in that some of its cisternae remain uninflated during all phases of growth. In Golgi aggregates without amplexi, the cisternae are characteristically uninflated (Fig. 121, 141). In those with amplexi, however, the cisternae are always differentiated, at least to some degree, into flat and inflated components (Fig. 154, 143). Table 15 summarizes the occurrence of the several types of Golgi apparatus among the species of *Tetracystis* and includes references to illustrative figures.

In the course of this investigation, some indication of the possible origin of the Golgi cisternae was uncovered. The protruding branches of the nuclear membranes, reported above and by others merely to encompass the Golgi apparatus, were observed by the writers also to give rise, apparently, to the cisternae themselves (Fig. 175–179). In this sequence of figures, it seems clear that the amplexus is giving rise to cisternae by a sort of budding process. It should be noted in these figures that the cisternae nearest the amplexus, and putatively, those most recently formed, are uninflated and closely packed, while those further away are somewhat inflated and seem to be separating. Furthermore, budding from the amplexus is limited to that portion adjacent to the most recently formed cisternae. These considerations are in harmony with the suggestion that the cisternae are arising from the amplexus by budding and orderly deposition of the products of this budding. No evidence was uncovered regarding the origin of cisternae in Golgi groups lacking distinct amplexi.

In this connection, Hodge and his co-workers (1956) suggested that a periodic continuity between the lamellar structures of the Golgi apparatus and the endoplasmic reticulum must occur. Whaley, Kephart, and Mollenhauer (1959) noted

TABLE 15. *Types of Golgi apparatus in Tetracystis*

I. Distinct amplexi present (cisternae differentiated)
   Tetracystis pampae (Fig. 157–3)
   T. aplanosporum (Fig. 154–5)
   T. excentrica (Fig. 143–9)
   T. texensis (Fig. 150–6)
   T. pulchra (Fig. 152–4)
   T. intermedium (Fig. 146–6)

II. Distinct amplexi absent (i.e., no distinct relationship between the Golgi system and the extension of the outer nuclear envelope).
   A. At least some cisternae inflated
      Tetracystis aggregata (Fig. 132–2)
      T. dissociata (Fig. 137–6)
   B. Cisternae not inflated
      Tetracystis isobilateralis (Fig. 131–4)
      T. aeria (C-6) (Fig. 123–7)
      T. aeria (Pa-3) (Fig. 124–2)
      T. illinoisensis (Fig. 141–3)

what appeared to be 1 or 2 isolated cisternal structures which might represent early stages in development of the Golgi apparatus. Moore and McAlear (1962) reported that in the fungus *Neobulgaria pura,* "the dictyosome[1] appears to be formed by a series of vesiculations of the outer membrane of the perinuclear cisternae that align to form a stack of sacs." Rhodin (1963) presented evidence on the origin of the Golgi bodies from the rough elements of the endoplasmic reticulum in animal tissue.

It is clear from these citations that there is some evidence for the origin of Golgi cisternae from the endoplasmic reticulum and from the amplexus, the latter probably representing a segment of the endoplasmic reticulum. One must be extremely cautious in correlating ultrastructure, which is static as viewed by the investigator, with function which is dynamic. However, study of *Tetracystis* has provided a possible indication of the function of the Golgi apparatus in algae, that of forming vacuoles. This same function has been ascribed to the Golgi apparatus in animal tissue by Rhodin (1963) and plant tissue by Marinos (1963). The evidence for this in *Tetracystis* is presented in Fig. 180–185 and their legends. In Fig. 183 one observes that the cisternae furthest from the amplexus have enlarged, presumably through the accumulation of fluid. In *Tetracystis aplanosporum,* the cisternae may become greatly enlarged and often separate from the Golgi system in pairs (Fig. 181) or in chains (Fig. 153–2). In other species of *Tetracystis,* the enlarged cisternae separate singly as shown in Fig. 179 and 152–4.

[1] Another appellation for the Golgi apparatus.

Finally, it should be stated that the present study has uncovered no evidence for a role of the Golgi apparatus in cell-plate formation, for cytokinesis in *Tetracystis* does not involve cell plate formation.

(5) The cell wall

Electron-microscopic observations of the cell wall layers of *Tetracystis* confirm the observations with light microscopy. Thus, the electron microscope reveals 2 layers in the wall, an outer, electron-dense layer, and an inner, less electron-dense, granular layer (Fig. 133–3). The outer layer often appears striated or lamellated which may indicate periodic deposition. This will be discussed in more detail under the section on cell division. As the cells age, the outer strata of the electron-dense layer sloughs off (Fig. 138–2).

For comparative purposes, the inner and outer wall thicknesses provided useful criteria for segregating actively growing *Tetracystis* species into 4 categories as is shown in Table 16.

The cell walls of species of *Tetracystis* appear to be similar to those of *Chlamydomonas* (Sager and Palade, 1957). These authors described a homogeneous, less electron-dense layer, about 400 Å thick, immediately adjacent to the plasma membrane. Exterior to this wall layer, they observed an electron-dense fibrillar layer which had a frayed appearance. This layer corresponded to the capsule (sheath) as seen with the light microscope.

TABLE 16. *Thickness of inner and outer wall layers in species of Tetracystis in the active growth phase*

I. Outer wall layer thicker than inner
T. aeria (C-6) (Fig. 121–1)
T. aeria (Pa-3) (Fig. 124–1)
II. Outer and inner wall layers of equal thickness, but both thin
T. aplanosporum (Fig. 153–1)
III. Outer wall layer thinner than inner
A. Inner wall layer very much thicker than the outer, forming uni- and bipolar thickenings
T. pulchra (Fig. 151)
T. excentrica (Fig. 143–6)
B. Inner wall layer only slightly thicker than the outer; polar thickenings absent
T. isobilateralis (Fig. 130–1)
T. aggregata (Fig. 133)
T. dissociata (Fig. 135–2)
T. illinoisensis (Fig. 141–4)
T. intermedium (Fig. 144–2)
T. texensis (Fig. 147–3)
T. pampae (Fig. 156–1)

Lang (1963), however, observed this duality of wall structure only in the zygote of *Astrephomene*. The vegetative cells of all 8 genera studied by Lang contained little or no material adjacent to the plasma membrane when fixed in $KMnO_4$. However, when fixed in OsO_4, her material showed a fibrous layer with dense granules immediately outside the cytoplasmic membrane.

Bisalputra and Weier (1963) found the inner wall layer adjacent to the cytoplasmic membrane in *Scenedesmus quadricauda* to be cellulosic. Outside this layer was a "middle lamella" bounded on both sides by an interface, and outside of the middle lamella was a pectic layer composed of a net of hexagonal configurations. The observations of Bisalputra and Weier of *S. quadricauda* seem to correlate with the writers' observations of the cell wall of *Tetracystis,* with the exception that pectic networks and a pectic spine are missing in *Tetracystis* species.

The thickness of the inner and outer wall layers of actively growing *Tetracystis* cells is a very constant and reliable criterion for species differentiation. Electron-microscopic studies have supported and augmented light-microscopic observations of wall structure. Cytochemical studies of the walls have not been included in the present investigation.

(6) Miscellaneous organelles

Other ultrastructural components of the *Tetracystis* cell were observed incidentally and are treated briefly below.

Endoplasmic reticulum (ER) was present in all species of *Tetracystis*. However, no consistent differences in its distribution and morphology could be discerned except, perhaps, for the amplexus which may be thought of as specialized ER. Just preceding cell division, ER abounds in the area immediately adjacent to the cytoplasmic membranes, particularly those formed during zoosporogenesis (Fig. 207–6) and occasionally during tetrad or diad formation (Fig. 190, 193, 199–4, and 201).

Tetracystis aplanosporum provided particularly interesting data with regard to contractile vacuolar organization. As described by Lang (1963), the contractile vacuole is delimited by a single membrane, and when fully distended, this layer is quite obvious (Fig. 186–2). When the contractile vacuole is partially (Fig. 187–5, 188–6) or completely collapsed (Fig. 186–1), this single membrane is folded radially. That segment of the contractile vacuole nearest the periphery of the cell has fewer folds in its limiting membrane (Fig. 187–5). Extensions from the outer nuclear envelope always seem to be associated with or in close proximity to the contractile vacuole (Fig. 187–4, 186–3, and 188–7).

3. Pyrenoid division

Although it is well known through light microscopy that pyrenoids divide during cellular replication (Timberlake, 1901; Lutman, 1910; Bold, 1931; Buffaloe,

1958; Bischoff and Bold, 1963), ultrastructural details of this process are not as well known. The pyrenoid has been observed to divide by fission, with light microscopy, giving rise to 2 or more pyrenoids. Many pyrenoids in division have been seen in electron micrographs. The details of pyrenoid division were observed with the greatest clarity in *Tetracystis isobilateralis*.

The lamellar elements of the chloroplast which penetrate the pyrenoid of *T. isobilateralis* have been studied before, during, and after pyrenoid division. In the non-division stages, the lamellar discs which penetrate the pyrenoid matrix assume a reticulate pattern with elongate interstices (Fig. 159–1). By the onset of division, the pyrenoid has achieved maximum size, and its lamellar discs re-orient and become parallel, extending across the entire pyrenoid matrix (Fig. 167–3). Still later, these parallel lamellae either are broken by elongation of the matrix, or break autonomously in a plane perpendicular to their long axis (Fig. 167–2). These breakages are not simultaneous.

Following cleavage of the parallel lamellae, 2 separate, double-disc systems within the pyrenoid penetrate perpendicular to the parallel discs and segregate the parallel disc system into 2 parts (Fig. 167–1, 169–8). The separated disc systems are still parallel (Fig. 168, 169). Figures 168 and 169 are from another section through the same pyrenoid and illustrate the contiguity of the intervening, perpendicular, double-disc systems with their respective segregated groupings of original parallel lamellae. Note also that the pyrenoid is more ellipsoidal, which may indicate a duplication or formation of additional matrix material at this stage before the initiation of fission.

Multiple stacks of chloroplast lamellae now begin to grow centripetally into the pyrenoid matrix (Fig. 168–5, 170). Starch formation is now initiated in the division zone (Fig. 170). The parallel relationship of the lamellae in the daughter pyrenoids persists (Fig. 170–15). At this stage, an ellipsoidal body has been observed several times between the daughter pyrenoids (Fig. 170–11). The significance of this body has not been resolved.

The writers consider pyrenoid cleavage to have been completed when the chloroplast lamellar intrusions have cut the daughter pyrenoids completely in half (Fig. 171–2). Whether or not the 2 sets of invaginating chloroplast lamellae anastomose or intertwine when they meet is unknown at present. When cleavage has been completed, 2 separate pyrenoid matrices can be discerned (Fig. 171), and starch synthesis becomes very active on the matrix interfaces of the daughter pyrenoids (Fig. 171). The lamellae in the daughter pyrenoids are still parallel to one another for the most part, but re-orientation soon occurs (Fig. 172–6).

The final stage in division of the pyrenoid consists of a reorientation of the parallel lamellae within the pyrenoid matrix to form the reticulate pattern of the "resting" pyrenoid.

Thus, when observed ultrastructurally, pyrenoid division appears to be more complex than light-microscopic observations have revealed. The role of the lamellae

within the pyrenoid matrix before, during, and after pyrenoid division is still unresolved. However, the characteristic orientations of the lamellae during division suggest that an orderly process, perhaps one analogous to nuclear division, may be involved in the division of the pyrenoid.

Since the discovery of DNA in the chloroplast (Ris, 1961; Ris and Plaut, 1962) it would seem possible that DNA might occur in the pyrenoid matrix, and, if so, that the pyrenoid may function as the genetic center of the chloroplast. In this laboratory, recent fluorescent microscopic studies have indicated that the pyrenoid of *T. isobilateralis* possibly contains RNA. Radio-isotopic studies with labeled cytosine and other cytochemical evidence will be required to confirm the suggestion made above.

4. Cell division

Since the nature of cell division has been emphasized in the characterization of the *Chlorosphaerales* (Herndon, 1958; Deason and Bold, 1960), and because so little was known about the nature of wall formation following cytokinesis, the writers employed the electron microscope to aid in the understanding of these processes, using species of the chlorosphaeralean genus *Tetracystis.*

It has been the writers' experience that it is very difficult to obtain large numbers of actively dividing cells at any given time without special preparation. Furthermore, the chance of observing a dividing cell with electron microscopy are limited, because of the relatively small spectrum of cells which can be observed at a given time. When grown on solidified BBM under standard conditions, the log phase of growth is relatively short (a 5–8 day span). Within 5–8 days after inoculation, the rate of cell division slows down, and the cells enter a stationary phase, accumulating large reserves of starch. Deficiency of nitrogen was found to be the primary cause for this. Therefore, the nitrogen concentration of BBM was increased 3-fold. Species of *Tetracystis* grown in the 3N[1] BBM remained in log phase up to 17 days following inoculation. When such cultures were harvested for electron-microscopic studies of cell division at 2 weeks, many more cells were found to be actively dividing.

Light plays an important role in determining which pathway will be followed, whether that leading to the formation of a zoosporangium or to a tetrad of nonmotile daughter vegetative cells. It will be recalled that cultures of *Tetracystis* were routinely maintained under a 12–12 hr photoperiod. If one were to examine actively growing cultures 6 hr into the light period, the highest frequency of division leading to the formation of nonmotile tetrads of daughter cells would be observable. However, when cultures are placed in continuous light for 1–3 hr, following an uninterrupted dark period of 5–8 hr, vegetative cells divide actively to form zoosporangia. Accordingly, an abundant supply of cells in various division stages could be obtained at will.

[1] 3-fold nitrogen increase as compared with BBM.

The first type of vegetative cell division to be described in *Tetracystis* involves division of a mature vegetative cell into a tetrad of vegetative cells with intervening diad formation. *Tetracystis isobilateralis* and *T. aggregata* most commonly undergo division in this manner. The chronological sequence of events, as observed by light microscopy, leading to the formation of a tetrad are shown diagrammatically in Text-fig. 3. In the second type of vegetative cell division, a diad stage does not intervene, but, instead, cytokinesis forms simultaneously 4 daughter cells (Text-fig. 2).

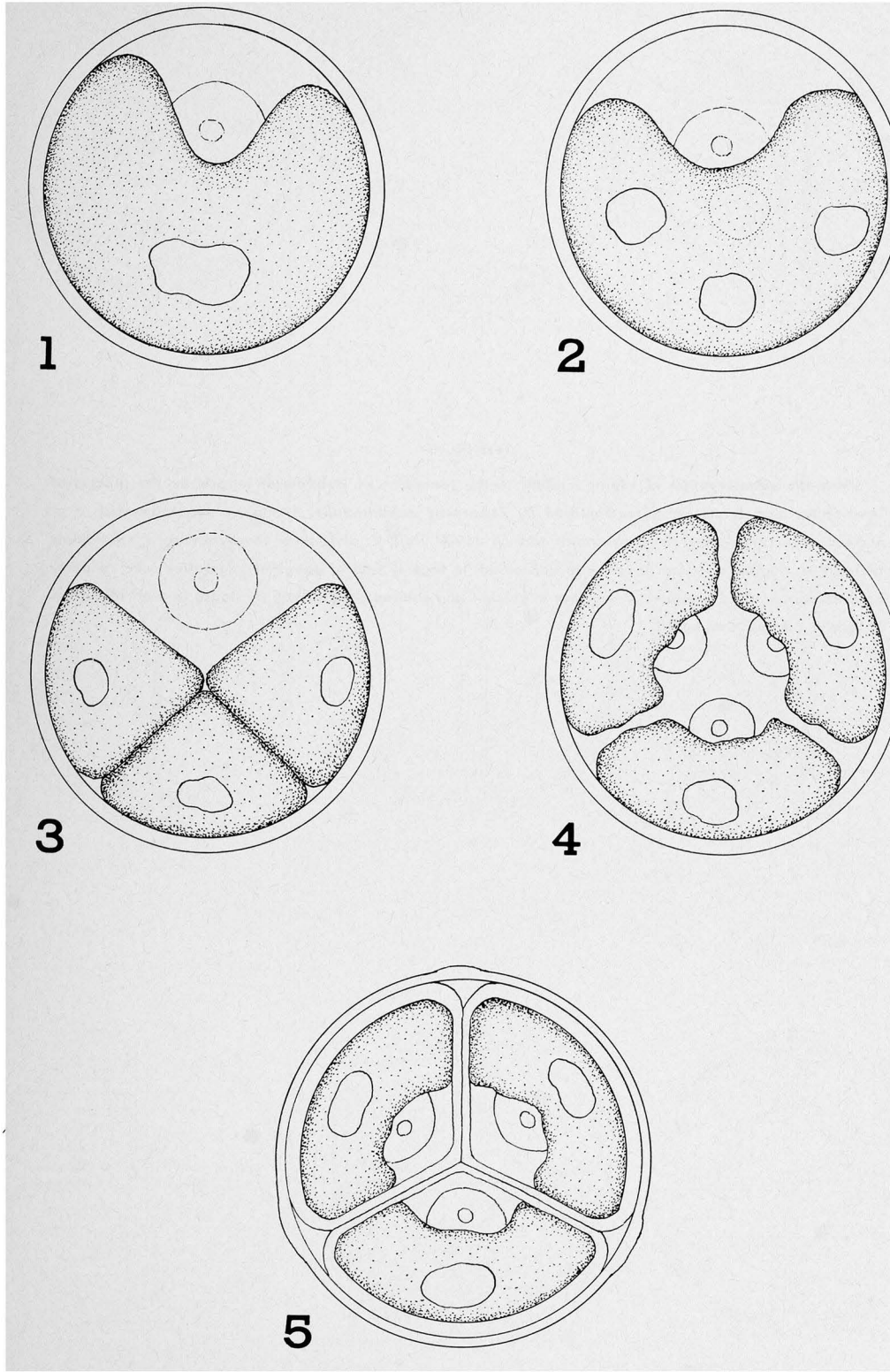
It is of interest to examine the ultrastructural events in both types of tetrad formation. Five of 12 species of *Tetracystis* will be discussed with reference to the 2 types of vegetative cell division.

Electron-microscopic observation of vegetative cell division into a tetrad, with intervening diads, will now be described. Examination of the wall structure of a mature vegetative cell of *T. aggregata* reveals 2 wall layers: an electron-dense, fibrillar, outer layer and an inner, less electron-dense, granular layer (Fig. 133–3). Following division of the pyrenoid, chloroplast, and nucleus (in that order) (Text-fig. 4), cytokinesis is initiated from the surface of the cell and progresses centripetally between the nuclei (Fig. 190–2). When the cleavage furrow reaches about midway into the cell, the inner wall begins to form in the area where the cleavage furrow had been initiated at the periphery (Fig. 191–4). The first sign of the presence of an inner wall layer is evidenced by a fine granulation image in $LiMnO_4$ fixation with Millonig's post-staining. Inner wall secretion appears to be progressive, following the cleavage furrow as it traverses the cell. The apex of the advancing cleavage furrow always seems to be surrounded by endoplasmic reticulum (Fig. 190, 195, 196). Golgi complexes are frequently observed in the perinuclear area during cleavage (Fig. 195–1, 190–1). However, their association seems to be less intimate with the cleavage furrow than is the association of the endoplasmic reticulum with the furrow. Figures 195 and 196 show 2 different sections through the same cell in which a cleavage furrow has cut through about 75% of the cell diameter. At the apex of the furrow, the Golgi apparatus is in sectional view of one figure (Fig. 195–1) and in surface view in the other (Fig. 196–2). Here no direct role of the Golgi apparatus in wall formation seems apparent.

It is of particular interest to note the presence of coarse granulation in the parent inner wall of the dividing cell shown in Fig. 191–3. Note that this granulation is present only in the inner wall of the parent cell (arrows) which is undergoing division to form a diad. A zone which separates coarse from fine granulation can be observed in the region in the transverse inner wall where cytokinesis began (Fig. 191–3). Thus, no coarse granulation appears in the transverse inner wall (Fig. 191–4), and no outer wall develops until such coarse granulation is present (Fig. 191). Other evidence for the relationship between coarse granulation of the inner wall and formation of the outer wall layer comes from examination of the "intercellular space" between the mature members of the diads (Fig. 192–5). Prior to the

Text-fig. 2

Schematic diagram of sequence of events leading to the formation of tetrads directly. 1. Mature vegetative cell; 2. pyrenoid division into 4 segments which may occur by successive bipartition or by direct fragmentation; 3. chloroplast division which may occur by successive bipartition or by direct fragmentation; 4. nuclear division; 5. cell wall deposition by invagination.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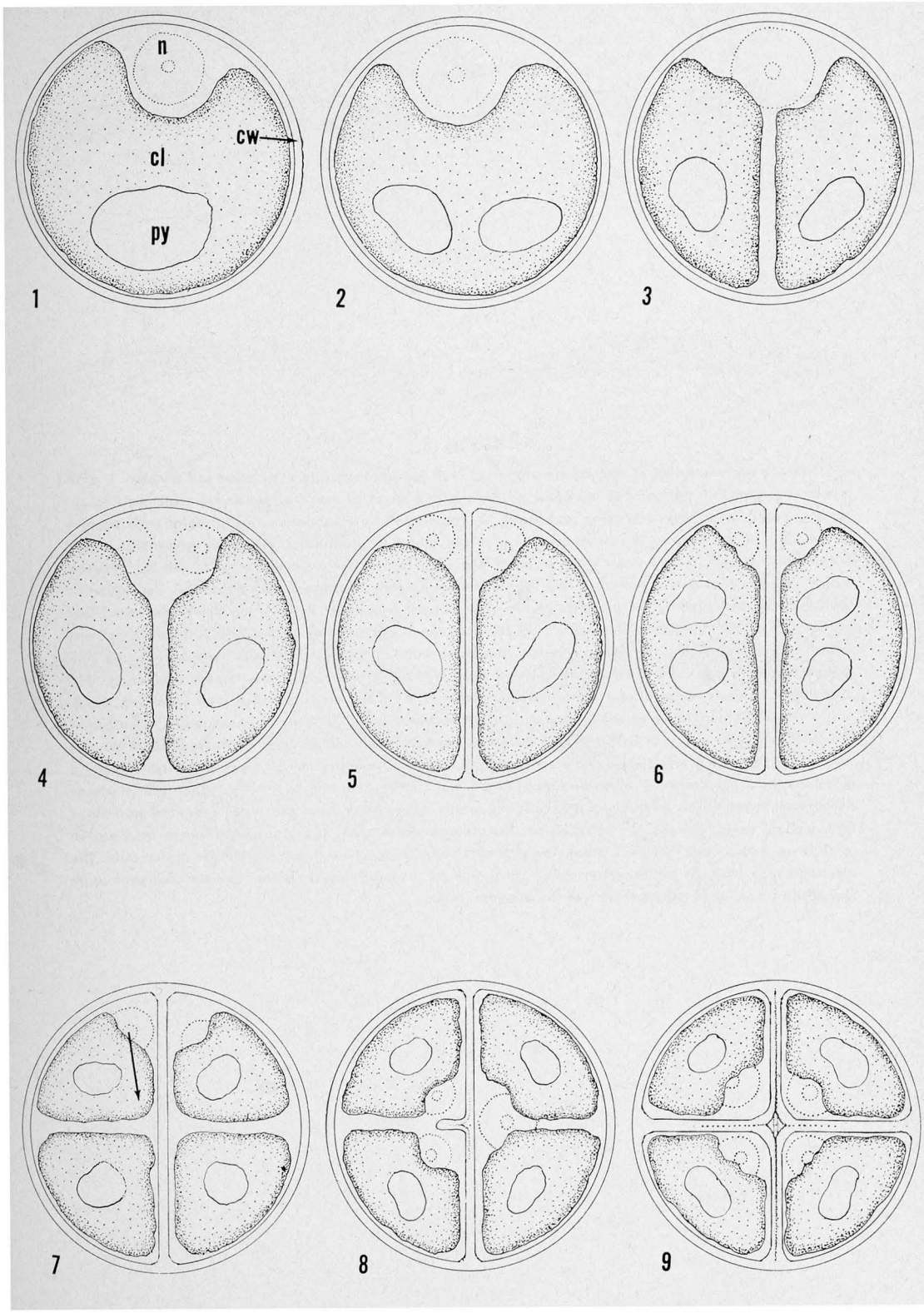
Text-fig. 3

Schematic representation of events leading to the formation of isobilateral tetrads by the process of intervening diad formation as exemplified by *Tetracystis isobilaterialis*. 1. mature vegetative cell: n = nucleus, cl = chloroplast, py = pyrenoid, and cw = cell wall; 2. division of the pyrenoid; 3. chloroplast division; 4. nuclear division; 5. cell wall deposition, to form a diad of daughter vegetative cells; 6. pyrenoid division in diad members; 7. nuclear migration and chloraplast division; 8. stages in wall formation to form the isobilateral tetrad.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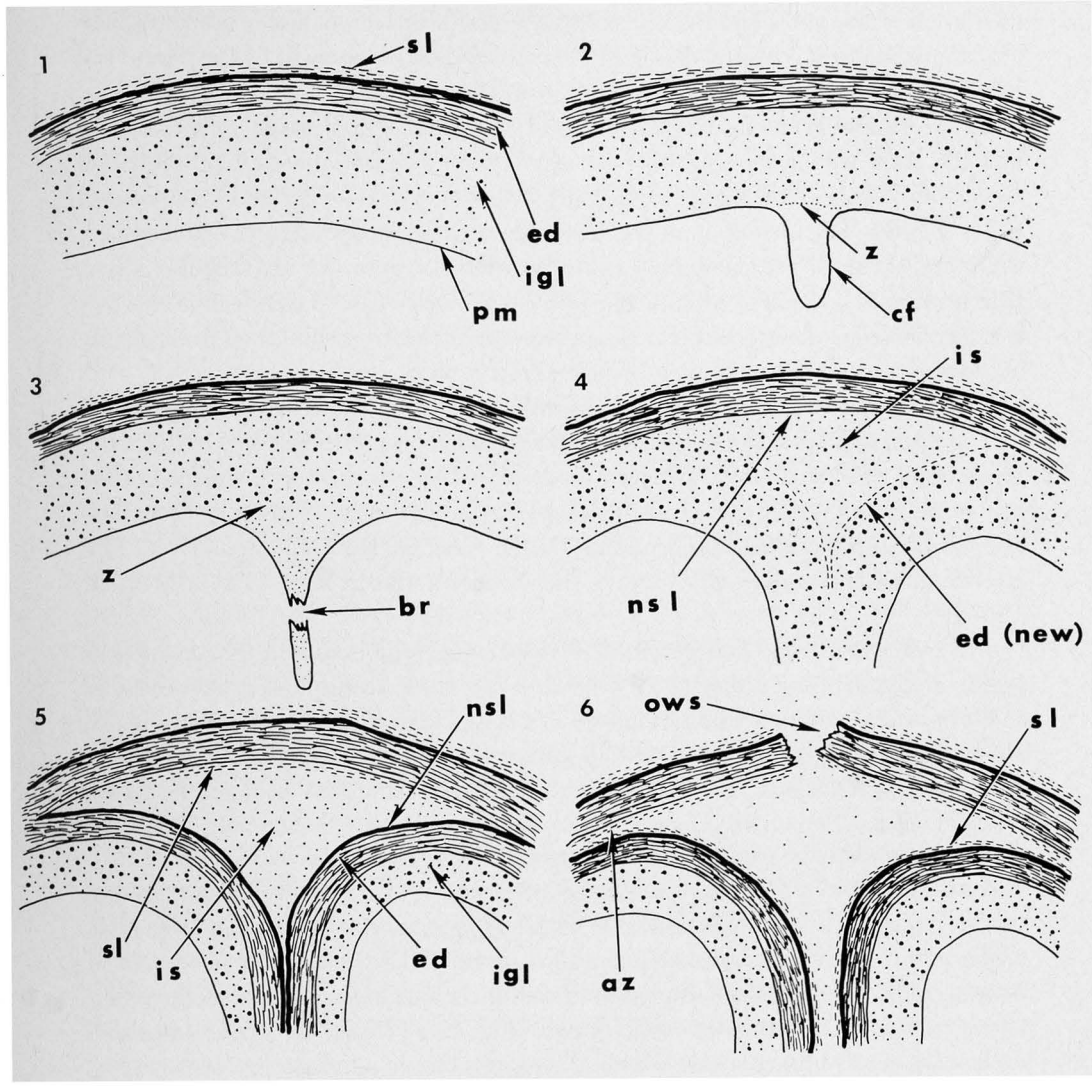
Text-fig. 4

Schematic representation of the various stages of wall formation during vegetative cell division. 1, wall layers of vegetative cell prior to division; sl = sloughing layer of the electron-dense wall (ed); igl = inner granular layer; pm = plasma membrane. 2, cleavage-furrow formation; no granulation is present in the apex of the furrow. The zone delimiting coarse and fine granulation originally present in parent inner wall is shown by z. 3, later stage in which cleavage furrow has cut across most of cell (br = break which indicates that only a portion of the furrow is shown). Note that inner wall deposition has begun in the furrow as indicated by the presence of fine granulation. A zone (z) delimits the newly deposited fine granulation of the daughter inner walls from the fine coarse granulation of the inner wall of the parent cell. 4, later stage of wall formation in which the inner walls of the daughter cells have become coarsely granulated; note also initiation of electron-dense wall (ed). An intercullular space is now evident (is) and coarse graulation is reduced here apparently by blockage from the electron-dense wall layer (ed, new). Nsl = non-sloughing layer of the parent-cell, electron-dense wall. 5, electron-dense wall formation of daughter cells now complete (ed) due to presence of coarse granulation of inner granular layer of daughter cell (igl). Coarse granulation completely absent in intercellular space (is) which accounts for sloughing in inner surface of parent-cell, electron-dense cell (sl). Nsl = non-sloughing layr eof daughter cell, electron-dense wall layer which is in close proximity to coarse granulation from the inner granular wall layer (igl). 6, final stage in vegetative cell division. The electron-dense walls of the parent cell have deteriorated to such an extent that they will break (ows = outer wall separation) releasing the daughter cells. The electron-dense walls of the daughter cells now slough (sl = sloughing layer) to form an abscission zone (az) which also aids in the separation of the daughter cell.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formation of the outer layers of the transverse walls, this intercellular space contains coarse granulation peripherally (Fig. 191–3), and the outer wall of the *parent* cell is being actively formed. Upon formation of the outer wall layer of the daughter cells, the coarse granulation disappears in the intercellular space (Fig. 192–5), and the outer wall layer (Fig. 192–6) of the parent wall begins to break down on *both* externally and internally, presumably because it is no longer being augmented (Fig. 192–6). The formation of the *new* outer wall layers of daughter cells appears, therefore, to block the passage of coarse granulation into the intercellular space, thus terminating augmentation of the outer wall layer of the parent cell in this sector. Accordingly, deterioration of the outer wall at the intercellular space probably accounts for tetrad dissociation as the cellular complex ages.

The origin of the wall layers just described has been most completely observed in *T. aggregata,* but it also occurs in similar fashion in *T. texensis* and *T. dissociata.* However, in *T. dissociata* a different mode of cytokinesis may occur instead of the unilateral invagination seen in *T. aggregata.* In *T. dissociata,* a plane of vacuolization occurs in the area to be occupied by the cleavage furrow (Fig. 197–4). Bilateral as well as unilateral cleavage can occur in this species (Fig. 197–3,–5; 198–3).

In *T. excentrica,* the number and activity of the Golgi bodies in the perinuclear region increase during cell division (Fig. 200–2). That the amplexus system may be thought of as a complex and unified structure is evidenced in Fig. 200–2 in which 4 Golgi apparatus can be observed to be encased within a common extension of the outer nuclear envelope. In this species, cytokinesis can occur unilaterally or bilaterally (Fig. 200–4). In *T. excentrica,* bipolar wall wall thickenings occur and these may possibly be explained by the unequal activity of inner wall formation.

A modification of the first type of vegetative cell division (2-step division to form tetrads) occurs in *T. isobilateralis.* Like *T. aggregata,* this species undergoes division to form a diad and thence proceeds to form a trial or tetrad. However, in *T. isobilateralis,* the granular component of the inner wall layer appears in the cleavage furrow as soon as the furrow is formed (Fig. 194). Thus, the outer wall seems to be deposited immediately behind the apex of the advancing furrow (Fig. 193–1). Note also that the intercellular space is devoid of coarse granulation which further supports the views discussed above (Fig. 193–2).

An examination of the newly formed daughter cell outer walls in *T. isobilateralis* reveals these layers to be very closely appressed, so that the space between their interfaces appears as a middle lamella (Fig. 193–4). This contiguity further supports previously published information (Herndon, 1958) which utilizes the contiguity of daughter cells with parent cells as a prime ordinal attribute delimiting the Chlorosphaerales.

Tetracystis isobilateralis may be useful in giving us insight into the origin of coarse granulation and its function in wall formation. The area of cytoplasm in the immediate vicinity of the advancing cleavage furrow (Fig. 194–6, 193–2)

contains an abundance of coarse granules as compared to other cytoplasmic areas. The only other areas containing an equal abundance of coarse granulation are the "vacuoles" (Fig. 193–5) which, perhaps, release these granules into the cytoplasm upon rupture. (The possibility of fixation artifact cannot be excluded here.) It is quite possible, therefore, that formation of the outer wall may be indirectly based on Golgi activity, for evidence has been presented earlier in this report that Golgi bodies may give rise to vacuoles.

The second type of vegetative cell division among *Tetracystis* species involves direct formation of a tetrad without intervening diad formation (Fig. 201). Here, the chloroplast and nuclei undergo 2 successive divisions prior to cytokinesis and subsequent wall formation. The writers have not yet seen enough cell divisions of this type to warrant a complete and detailed description. However, information of some value has been gained from observations of cell divisions in *T. aeria* (Pa–3). Outer wall formation in *T. aeria* is similar to that in *T. isobilateralis* in that the outer wall is formed immediately behind the apex of the advancing cleavage furrow. One of the major differences in the second type of vegetative cell division is the path the cleavage furrow will take, once it has been initiated, after the cell has become quadrinucleate. If thickness of the newly deposited outer wall layer is a magnification of a time-deposition relationship, then the later-formed layers may be detected. Note in Fig. 201–5,–6 that 2 such thick outer walls are present. Thus, 2 simultaneous cleavages could have arisen from the cell surface (arrows) and developed centripetally (Fig. 201–5,–6) and thence centrifugally (Fig. 201–7,–8) to the surface of the other pole of the cell (note the thinner outer wall layers in the centrifugal cleavages).

In view of the foregoing, the type of vegetative cell division which forms tetrads directly may be thought of in terms of a limited progressive cleavage, with a considerably more specific pattern than is exhibited in other algae which have many more nuclei per cell (e.g., *Protosiphon*).

Vegetative cell division in *Tetracystis,* then, is effected by cytokinesis followed by the formation of an inner wall in the plane of the cleavage furrow. When coarse granulation is present (*T. aggregata* and *T. isobilateralis*), an outer wall layer is formed. Outer wall formation may be delayed until the cleavage furrow and subsequent inner wall deposition have been completed (*T. aggregata*), or outer-wall formation may occur simultaneously with cleavage and inner wall formation (*T. isobilateralis*). In all species, the outer walls of newly formed daughter cells remain closely appressed to the parent cell wall immediately following division, thus supporting Herndon's (1958) statements on wall contiguity. However, daughter-cell separation may be early or late, depending on the rapidity of outer wall breakdown at the intercellular spaces of a given species.

5 Zoosporogenesis in *Tetracystis* and *Chlorococcum*

During the present electron-microscopic investigation of *Tetracystis,* the oppor-

tunity became available to examine the ultrastructural events leading to the formation of zoospores. It will be recalled that *Tetracystis* may follow 2 pathways of cell division, one leading to the formation of nonmotile daughter cells, and the other leading to the development of zoospores (Text-fig. 1). As has been discussed previously, the existence of the above-mentioned alternatives in *Tetracystis* delimits it from the genus *Chlorococcum*. In *Chlorococcum,* only 1 of the pathways of cellular division is present, namely, zoosporogensis. Since both *Tetracystis* and *Chlorococcum* can form zoospores, the writers wished to compare zoosporogenesis ultrastructurally in at least 1 species of these 2 genera representative of the *Chlorosphaerales* and *Chlorococcales,* respectively, and also to compare zoosporogenesis with vegetative cell division.

The first indication of the initiation of zoosporogenesis in a mature vegetative cell, as observed by light microscopy, is the "disappearance" of the pyrenoid, accompanied by an increase in density of the chloroplast (Fig. 69). *Tetracystis aplanosporum* was used to demonstrate ultrastructural changes in the pyrenoid and chloroplast leading to the formation of zoospores. During interphase and growth of the mature vegetative cell, the pyrenoid is a conspicuous body in the chloroplast and usually is located in the center of the cell (Fig. 153). Aggregates of starch plates surround the pyrenoid matrix during this phase (Fig. 153).

When a vegetative cell is destined to undergo zoospore formation evoked by the environmental conditions described on page 58, the pyrenoid becomes segmented by the dividing chloroplast (Fig. 202–205). The furrows which divide the chloroplast also appear ultimately to divide the pyrenoid first into hemispheres (Fig. 203), and then into a number of segments which foreshadow the number of zoospores which will be formed (Fig. 205). In contrast, it will be recalled that pyrenoid division during vegetative cell division is significantly different (Figs. 173, 174). It is of interest that in zoosporogensis, starch synthesis at the periphery of the pyrenoid matrix is greatly reduced during pyrenoid division. While pyrenoid starch may be reduced during this phase, non-pyrenoidal starch seems to increase. Simultaneous reduction of pyrenoid starch and increase of non-pyrenoidal starch may account partially for light-microscopic observations of the supposed "disappearance" of the pyrenoid during this phase of zoosporogenesis. While light-microscopic observations have been inadequate to reveal the fate of the pyrenoid, electron microscopy indicates that the pyrenoid does not actually disappear during division as Fig. 202–205 clearly show. Evidence for the presence of the pyrenoid, during and after its division, is based upon the characteristic presence of pyrenoid tubules associated with pyrenoid stroma (Fig. 204–2). Thus, pyrenoid division during zoosporogenesis appears to differ little from that process in vegetative cell division, except for the reduction of pyrenoid starch and multiple simultaneous cleavages of the chloroplast which divide the pyrenoid into fragments, one destined for each zoospore.

Figure 205 shows a later stage in zoosporogenesis of *T. aplanosporum* in which

the chloroplast has nearly completed division. At this stage, the lamellar structures of the chloroplast are more numerous and more densely aggregated than in the resting cell. The lamellar proliferation just noted represents a period of maximum chloroplast lamellar synthesis and pigment formation. In support of this statement, light-microscopic observations show a deeper coloration and increased density of the chloroplast during early zoosporogenesis.

A major cytoplasmic change during early zoosporogenesis is an increase in quantity and activity of the Golgi apparatus (Fig. 204–3). The activity of the Golgi system is evidenced by the greater degree of vacuolization of certain cisternal components in the dividing cell (Fig. 204–3) than in the interphase cell (Fig. 153).

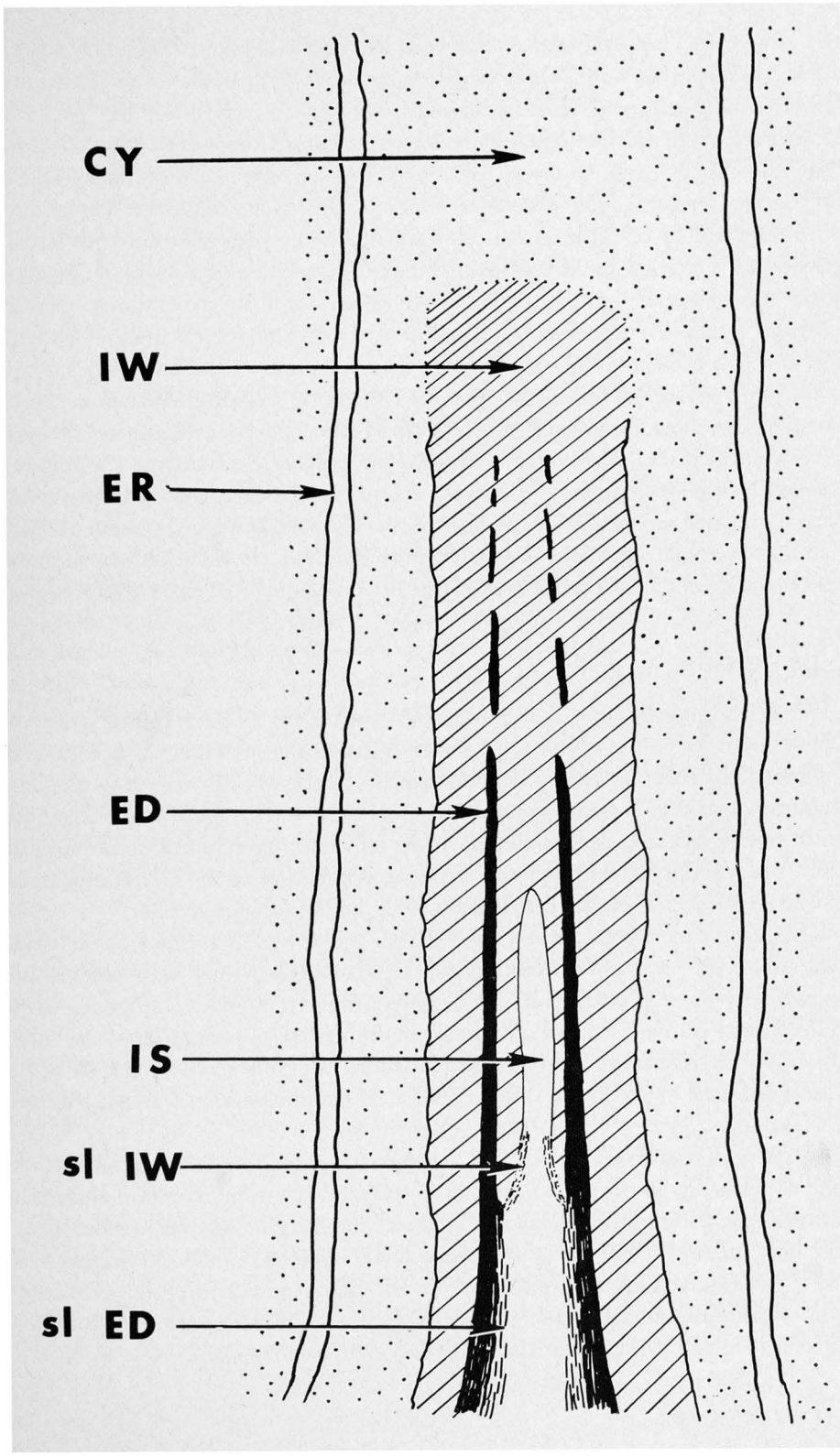
Following pyrenoid, chloroplast, and nuclear division, events leading to the formation of the zoospore wall are initiated and will now be described in detail for *Chlorococcum multinucleatum* and *Chlorococcum* sp: (tetra, rough) (Text fig. 5). Here, the endoplasmic reticulum, unlike that in vegetative cell division, appears to play a major role in wall formation leading to zoospore development. Branches of the ER develop at the surface around each protoplasmic segment destined to become a zoospore (Fig. 206–2, 207–6). There is continuous cytoplasm between the immature zoospore segments bounded by ER. Later, a common wall layer is secreted peripherally to the endoplasmic reticula of adjacent zoospores (Fig. 206–1). Shortly thereafter, adjacent, electron-dense wall layers, one from each zoospore, are formed, these separating the common wall layer which had been previously deposited (Fig. 207–8). These electron-dense layers are destined to become the outer wall layers of the mature zoospores. Since the common wall layer ceases to be actively deposited outside of the developing electron-dense wall layers, the common wall layer (IW) breaks down, releasing the individual zoospores shortly thereafter (Fig. 208, 209). Remains of the earlier-deposited common wall (IW) adhere to the electron-dense walls of the newly-separated zoospores but are soon sloughed off, exposing now only the outer, electron-dense layers. Prior to this loss of the common wall, the electron-dense walls were bounded by distinct interfaces on both surfaces. However, when the common wall has been sloughed off completely, exposing only the outer, electron-dense layers, the latter also begin to slough. The rate of loss of electron-dense wall material is less than the rate of is deposition.

An interesting feature of zoosporogenesis is the very early formation of flagella and stigma, even before zoospore wall formation has been completed (Fig. 207–4,–5). Impending zoosporogenesis may, therefore, be distinguished from impending division to form tetrads of nonmotile vegetative cells even before cytokinesis and wall formation have been initiated. Other investigators have reported that flagella and stigma arise *de novo* in each zoospore (Bold, 1951).

A most significant, comparative, ultrastructural feature of taxonomic value is the relation of the parent zoosporangial wall to that of the zoospore. It will be recalled that in vegetative cell division, the electron-dense wall layers of the daughter cells

Text-fig. 5

Diagrammatic representation of cell wall formation during zoosporogenesis in *Tetracystis* and *Chlorococcum*. The earlier events begin at the top of the illustration and progress as viewed downward. CY = cytoplasm; IW = common inner, electron-transparent wall layer; ER = endoplasmic reticulum, delimiting protoplasmic segments destined to become zoospores; ED = electron-dense wall layer, deposited, peripherally to the inner wall layer; IS = intercellular space, formed by deterioration of common wall layer (IW); sl IW = soughing inner wall layer remains of the earlier-deposited, common, inner wall layer; sl ED = sloughing electron-dense wall layer which begins to slough only when the remains of the common inner wall layer have eroded away.


CY
IW
ER
ED
IS
sl IW
sl ED

arise adjacent to the equivalent, electron-dense layers of the parent (Fig. 192–7) cell wall. The parent wall is composed only of the electron-dense layer, and rupturing of this outer layer will release the daughter cells.

By contrast, the zoosporangial (parent) wall is composed of both outer, electron-dense and inner, granular layers (retained from its vegetative precursor) (Fig. 208–1,–2). Here, the outer wall layer of the zoospore is not deposited contiguously to the outer wall layer of the parent cell wall. This fact represents a major difference between the 2 types of cell division which have been described and should be given proper emphasis in delimiting the order Chlorococcales from the Chlorosphaerales.

Zoosporogenesis is significantly different from vegetative cell division with respect to: (1) the simultaneous, multiple fragmentation of the pyrenoid; (2) reduction of pyrenoid starch during pyrenoid division; (3) proliferation of chloroplast lamellae during division; (4) increase of chloroplast starch during division; (5) early presence of stigmata and flagella; (6) apparent relation of endoplasmic reticulum in wall formation; (7) early secretion of the zoospore electron-dense wall layer; (8) early separation of division products (zoospores) as a result of early electron-dense wall secretion and/or maturation; and (9) lack of association of outer wall layers of the zoospore with the outer wall layer of the zoosporangium.

C. DISCUSSION

The electron-microscopic studies reported above had as their primary purpose elucidation of the nature of chlorosphaeralean (as compared with chlorococcalean) cytokinesis and wall organization and formation. As the work progressed, secondary comparative data of significance were accumulated with respect to the organization of the chloroplast as a whole and of its lamellae; pyrenoid structure and division; mitochondrial form; possible origin and function of the Golgi apparatus; cell wall structure; vegetative cell division, and, finally, zoosporogenesis.

With respect to vegetative cell division, an inner wall is formed in the plane of an advancing cleavage furrow, and an electron-dense, outer wall layer is subsequently deposited. The electron-dense wall of the parent cell is contiguous to the electron-dense wall layer of the daughter cell at the time of formation of the latter, thus supporting Herndon's (1958) emphasis of contiguity of the cell wall of the division products in vegetative cell division. Cell plate formation was not observed in *Tetracystis*.

Zoosporogenesis was studied comparatively with vegetative cell division and was found to differ significantly, particularly in the mode of wall formation and in the relation of the parent cell wall layers to those of the daughter cells. Zoosporogenesis is the only method of cell division present in the Chlorococcales. Vegetative cells of the Chlorosphaerales may undergo both zoosporogenesis and vegetative cell division. Furthermore, the differences between these 2 orders are brought more sharply into focus by ultrastructural studies of cell division.

Pyrenoid division during vegetative cell division was studied in detail in *Tetracystis isobilateralis*. As observed by the light microscope, the pyrenoid divides by fission. However, the process is more complex ultrastructurally in that the lamellar structures penetrating the pyrenoid matrix assume parallel orientation during division and subrandom orientation during interphase. Final division of the pyrenoid is accomplished by centripetal growth of chloroplast lamellae in the division zone between the daughter pyrenoid matrices. In *Tetracystic aplanosporum* and *T. aeria* (C–6), however, the pyrenoid is cleaved by the chloroplast-limiting membrane.

Electron-microscopic study has revealed that parent cells transmit fragments of the original pyrenoid to the zoospores they form, so that they do not, in fact, arise *de novo* as sometimes reported on the basis of light microscopy. The stigmata and flagella, on the other hand, clearly do arise *de novo*.

The species of *Tetracystis* were studied comparatively for consistent differences in organelle structure. The structure of the chloroplast, as observed electron microscopically, greatly augmented light-microscopic observations. The internal structure of the chloroplast was useful in differentiating *Tetracystis* species into groups based on the pattern and number of associated lamellar discs. *Tetracystis pampae* has a most striking internal chloroplast organization in that the chloroplast lamellar discs are associated always in 3's. *Tetracystis aplanosporum* also has a distinct pattern of 1–2 discs alternating with groups of 3–5 discs. Other species have distinct patterns of lamellar disc stacking, but the number of lamellar discs per stack is somewhat more variable. The remaining species of *Tetracystis* were grouped into a category in which both the pattern and number of associated chloroplast lamellar discs were variable.

Of all the organelles, the pyrenoid provided the most reliable and striking ultrastructural differences. Pyrenoids of *Tetracystis* are surrounded either by 2 starch grains or by many starch grains. Those pyrenoids with 2 starch grains have no lamellae penetrating into the pyrenoid matrix except for a convoluted single-to-double disc system between the starch grain and the pyrenoid matrix. Pyrenoids which fall into the above category are always ellipsoidal and can be easily differentiated also by light microscopy with respect to shape. Pyrenoids with many starch grains are of 3 types: (1) those with single tubules penetrating the pyrenoid matrix; (2) those with a double disc system of lamellae penetrating the matrix; and (3) those with a triple disc system of lamellae penetrating the matrix. Pyrenoids with a double disc system can be further subdivided on the basis of the disc pattern within the pyrenoid and the pyrenoid size itself.

Mitochondrial differences were few among the species of *Tetracystis,* but when such differences were present, they were striking. *Tetracystis isobilateralis* and *T. aggregata* were the only 2 species with compressed mitochondria, while all other species of *Tetracystis* contained mitochondria organized as cylinders of variable dimensions. *Tetracystis isobilateralis* could be easily differentiated from all other

species on the basis of frequent branching of the large, compressed mitochondria.

Cell wall ultrastructure provided useful criteria for augmenting light-microscopic observations of cell wall thickness of actively growing cultures, and, in addition, for studying comparatively, thickness of inner and outer wall layers. Both isolates (C–6 and Pa–3) of *Tetracystis aeria* were distinct from all other species in that the outer, electron-dense wall is thicker than the inner wall layer. In *T. aplanosporum,* both inner and outer wall layers are thin and equal in thickness. In *T. pulchra* and *T. excentrica,* the inner wall layer is often very much thicker than the outer, electron-dense layer, and this may be observed in light microscopy as internal unipolar and bipolar wall thickenings. The remaining species of *Tetracystis* are more variable in wall thickness and could not be further differentiated except that the inner wall layer is only slightly thicker than the outer.

The Golgi apparatus was studied comparatively and provided useful data for segregating *Tetracystis* species into 2 categories: (1) those with Golgi bodies with distinct amplexi, the cisternae of which are often inflated furthermost from the encompassing element of the amplexus; and (2) those with Golgi without distinct amplexi, cisternae of which were rarely observed to inflate.

A possible origin of the Golgi apparatus was observed in those species in which the Golgi bodies have amplexi (category 1 above). Here, the Golgi cisternae seem to be formed by a budding process of the associated, encompassing portion of the amplexus (which may be specialized endoplasmic reticulum). Such budding activity seems to be present in both resting and dividing cells of actively growing cultures. Evidence of origin of Golgi cisternae was not so clear in Golgi apparatus without distinct amplexi.

Although function cannot always be completely understood from structure, a possible function of the Golgi cisternae in the role of vacuole formation was noted among those species which have distinct amplexi. The cisternae seem to have a period of activity of vacuole formation which is greatest during cell division. Contents of the vacuole are unknown, but the structure of the vacuole in $LiMnO_4$ preparations seems to indicate that some substance has been removed by the fixation, leaving behind a cavity limited by a single membrane.

Other organelles were examined comparatively. Endoplasmic reticulum provided no reliable comparative data. However, the presence and orientation of the ER during zoosporogenesis may signify its specialized role in wall formation. Contractile vacuoles provided no useful comparative data; their structure and possible association with the nucleus in *Tetracystis aplanosporum* were observed. Stigma and flagella were not studied in this investigation.

Thus, species of *Tetracystis* can be segregated on the basis of ultrastructural organization. The same species which fit into a certain category on the basis of one organelle system, also may be grouped with respect to similarity of other organelles (Table 17). On this basis, it would be reasonable to assume that the majority of the ultrastructural differences observed are significant and that they characterize a given species or a group of closely related species.

TABLE 17. *Groupings of Tetracystis species on the basis of similarity of ultrastructure*[a]

Organelle	Groupings				
Mitochondrion	*T. iso.* *T. agg.* *T. diss.*		*T. aeria* (C-6 & Pa-3) *T. excen.* *T. illin.* *T. pam.*		*T. pul.* *T. tex.* *T. aplano.* *T. inter.*
Cell wall		*T. aeria* (C-6 & Pa-3)	*T. pul.* *T. excen.*	*T. aplano.*	*T. iso.* *T. agg.* *T. diss.* *T. illin.* *T. inter.* *T. tex.* *T. pam.*
Pyrenoid	*T. iso.* *T. agg.*	*T. aeria* (C-6 & Pa-3) *T. diss.* *T. illin.*	*T. excen.* *T. pul.* *T. inter.* *T. tex.*	*T. aplano.*	*T. pam.*
Chloroplast mass	*T. iso.* *T. agg.* *T. diss.*	*T. aeria* (C-6 & Pa-3)	*T. excen.* *T. pul.* *T. inter.* *T. tex.* *T. illin.* *T. pam.*	*T. aplano.*	
Chloroplast internal	*T. iso.* *T. agg.* *T. diss.*	*T. aeria* (C-6 & Pa-3) *T. illin.*	*T. excen.* *T. pul.* *T. inter.* *T. tex.*	*T. aplano.*	*T. pam.*
Golgi apparatus	*T. agg.* *T. diss.*	*T. aeria* (C-6 & Pa-3) *T. illin.* *T. iso.*	*T. excen.* *T. pul.* *T. inter.* *T. tex.* *T. pam.* *T. aplano.*		

[a] This table emphasizes groupings of the species. For details of the organelles of the several species, see text and tables.

IV. Immunochemical Studies with *Tetracystis* and *Chlorococcum*

A. INTRODUCTION

The science of serology or immunochemistry, with its latest developments in technique, is now firmly grounded in medicine, zoology, and microbiology. Serology has received less attention in many areas of botany because there has been no real need for its use in many instances. This has been particularly true where taxonomy has required no supplementary criteria in the identification process, as is considered by many to be the case in the so-called "higher plants." On the other hand, certain groups, namely, the bacteria, pathogenic fungi, and plant allergens, have received more attention by the serologist either because these organisms are not so well-defined morphologically or because of the medical importance of their pathogenicity to man. It must be added that most botanists have not been trained in serology which is regarded principally as a zoological or microbiological technique. Finally, lack of knowledge and bias against its use by botanists may also account for the slow development of serological techniques in botany.

Plant serology received considerable attention in the first quarter of this century during which the Königsberg school of plant serology published many works, culminating in the "Stammbaum" or phylogenetic tree of the plant kingdom (Mez and Ziegenspeck, 1926). This work was subsequently repeated (using different techniques) by the Berlin group. Failure of the Berlin group to achieve the same results as the Königsberg school led to considerable disagreement. Subsequently, the reliability of serology as a tool for phylogenetic and systematic studies has been questioned. Only recently, however, have the techniques of serology been applied to plant taxonomy with renewed interest and promise. Among others, the works of Gell, Hawkes and Wright (1960) and Lester (1964) have shown the value of serology in "higher plant" systematics using the newer techniques of double-diffusion (Ouchterlony, 1948b) and immunoelectrophoresis (Grabar, 1959).

Serological studies of the algae have been fewer than those of angiosperms. According to Mintz and Lewin (1954), Rosenblat-Lichtenstein (1913) reported serological differences between a green strain of *Chlorella protothecoides* Kruger and a spontaneous mutant of the same species which had reduced pigmentation.

In 1928, Mary Elmore was able to distinguish *Euglena, Chlorella,* and *Chlamydomonas* by complement fixation tests; however, she could not differentiate different strains of *Euglena* on that basis. In 1935, Mary Elmore Sauer reported further work based on 6 strains of *Euglena gracilis* in which she was able to divide them into 2 serological groupings of 3 strains each. These categories were based on the loss of motility and complete sedimentation, as against no loss of motility and no sedimentation, when the organisms were placed in an antiserum to one or more of the strains at dilutions up to 1:1000. These serological groupings corre-

lated exactly with those in which the growth characteristics had been the criteria for their separation.

Provasoli, Pintner, and Haskins (1951) failed to produce antigenic distinctions between mating types of *Chlamydomonas moewusii;* however, serological differences between *C. moewusii, C. chlamydogama,* and *C. reinhardti* were readily detected. No mention was made of the methods employed to distinguish the species.

Mintz and Lewin (1954) prepared flagellar suspensions of the plus and minus wild-type cells and paralyzed mutants of *Chlamydomonas moewusii,* and by complement fixation, were unable to distinguish between the 2 wild mating types or between the wild-type and paralyzed mutants.

Flagellar studies have been made with other members of the Volvocales. Among these, the work of Coleman (1963) deserves special attention. Coleman studied immobilization, agglutination, and agar precipitation of antibodies to flagella of the mating types of *Pandorina.* The antisera prepared against *Pandorina* exhibited syngen specificity, i.e., syngens (sexually isolated populations within the species) maintained their antigenic specificity, even though they were isolated from diverse geographical localities. However, serological mating-type specificity was not achieved with *Pandorina.*

The writers became interested in the immunochemical approach to the taxonomy of chlorococcalean and chlorosphaeralean algae during the summer of 1962 when they provided Mr. Charles Sweet with axenic cultures of *Chlorococcum* and *Tetracystis* species for injection into rabbits. The antisera produced by Sweet, however, were weak in precipitating antibodies.

While most of the recent immunochemical investigations have been concerned with flagellar antigens, the somatic or whole-cell antigens have, for the most part, been little studied. For this reason, the writers used whole-cell antigens because it was considered that they might be more useful for taxonomic purposes, since greater diversity and quantity of antigen types would be obtained. Furthermore, the recent development and perfection of double-diffusion and immunoelectrophoresis have provided analytical tools of incomparable resolution for protein systems. These tools have been used here with considerable success with algae.

B. MATERIALS AND METHODS

1. Preparation of cells for extraction

During the early phase of this investigation, algal material was prepared by growing it in liquid BBM in 125-ml Erlenmeyer flasks under standard conditions of culture. However, the yield of algal cells was too low to provide a quantity sufficient for subsequent breakage and protein extraction. Therefore, another method was introduced for growing algal material. Petri dishes (20 × 100 mm) containing 1.6% BBM agar with 3 × nitrogen concentration were inoculated with algae

(see page 9) and grown under standard conditions for 2–3 weeks, after which the algae were harvested. On the day of harvesting, each plate was carefully screened macroscopically and microscopically for possible fungal and/or bacterial contamination, because all cultures to be used of necessity had to be axenic. The cultures were harvested by gently scraping the algae from the agar surface with the edge of a sterile microscope slide. The algal mass was then transferred either to a beaker for storage in a freezer, or directly into a chilled mortar (−15° C) for immediate processing.

A number of different methods were tested for breakage of the algal cells. The following techniques were unsuccessful in achieving satisfactory breakage: (1) motar with sand, 5° C; (2) mortar with alumina, 5 °C; (3) Mickle Cell; (4) Ribi Cell Fractionator; (5) Bransen ultrasonic probe; and (6) tissue homogenizers, both Teflon and glass.

The method which eventually proved to be the most satisfactory for cell breakage was the acetone-powder method (Stafford and Magaldi, 1954; Colowick and Kaplan, 1955). Early experience with this method gave only mediocre results; however, certain modifications, which will be discussed below, resulted in excellent algal cell breakage.

The acetone-powder method has several advantages over the other methods described above, namely: (1) very efficient cell breakage with *Tetracystis* and *Chlorococcum;* (2) acetone-soluble materials such as chlorophyll, lipids, and water were removed, leaving the dehydrated proteins and cellular debris in the preparation; and (3) the complete process was carried on below 0° C until the preparation was in the form of a dry powder.

Since the acetone-powder method was so successful in breakage of the algal cells, and because proteins of good antigenicity could be obtained in this manner, a somewhat detailed description of the process will be presented here. A block (6″ × 6″ × 2″) of dry ice was placed in an enamelled pan (6″ deep) on top of layers of paper towels for insulation, and a Coors' No. D-27 mortar was placed on the block for chilling (10–15 min) to about −15° C. The pestle was placed in the mortar so that it too would be chilled. Then the algal mass, either from the frozen pellet of storage material, or the mass scraped from a Petri dish, was added to the chilled mortar and allowed to freeze solid (1–2 min). When all of the Petri dishes had been harvested and when the material had become frozen, the algal pellets were gently crushed into a coarse powder. Cheese cloth usually was placed over the mortar in order to prevent loss of flying material during this tedious process. When the material had been ground to a state fine enough that no particles would be knocked from the mortar, the cheese cloth was removed and the particles ground to a fine powder. Enough acetone was then added to form a very thick paste.

If too much acetone was added, a slurry would be formed, and this would need to be evaporated to a paste before efficient cell breakage would occur. When the

proper consistency[1] had been achieved, the mass was ground with considerable pressure on the pestle in order to push the mass over the bottom surface of the mortar. A swirling action with the pestle tended to push the mass on the sides of the mortar, and when this occurred, the material was scraped back into the bottom, and the grinding process continued. At this stage, periodic checks for efficiency of cell breakage were made by collecting a small amount of the paste on a chilled spatula and transferring it to a microscope slide for immediate examination.

When 80–90% of the cells had been broken, 40–50 ml of chilled 99.9% acetone (−15° C) were added to the paste, and, with the action of the pestle, a slurry was formed. In preparation for this slurry, a 6″-disc of Whatman No. 1 filter paper was prepared to fit into a glass funnel, and chilled acetone was added to cool the system. Then the slurry of algal material was poured onto the filter and placed in a freezer (−10° C). Fifty–100 ml of additional chilled acetone were added to further extract the fat-soluble material, chlorophyll, etc., until a clear filtrate was obtained. Since proteins are insoluble in acetone, they and the cellular debris did not pass through the filter. When most of the acetone had passed through the filter, it was folded, stapled, and placed in a freezer with a frost-clearing system and allowed to dry for 24–48 hr. Then the dry filter paper was brought to room temperature, and the powder scraped from the surface. The dry material was then placed in a Coors' No. 520–0 mortar and pestle (which was dry and at room temperature) and lightly ground to a very fine powder. The powder was weighed, placed in an air-tight container, and stored at −10° C until needed for extraction.

2. Extraction procedures

The following extraction solution was employed for all extraction procedures, namely, 0.15 M NaCl buffered with a 0.01 M sodium phosphate buffer, pH 7.5. This was prepared as follows:

NaCl	8.7 g
$NaH_2PO_4 \cdot H_2O$	0.2208 g
$Na_2HPO_4 \cdot 12H_2O$	3.0085 g
Deionized H_2O	1000 ml

It was discovered only later in this study that the stability of algal proteins as potent antigens decreased upon repeated thawing and re-freezing of the extracted powder (8–10 times) and upon prolonged storage (2–6 months) while in solution. Therefore, it has been found much more desirable to extract only the amount needed, either for injection or testing purposes, and to maintain the antigen in powdered form at −10° C.

[1] The consistency of the algal mass is just right for efficient cell breakage when the cells will form a homogeneous layer which adheres to the bottom of the mortar when gentle-to-moderate pressure is applied to the moving pestle.

All extractions were carried out at 5° C from 2 to 24 hr. A ratio of 0.1 g dry powder to 1.0 ml extraction solution was employed for most extractions. The protein concentrations of this ratio in all species tested varied from 2.3 to 17 mg/ml. Several experiments were performed to determine the precipitin reactivity over a wide range of concentrations (Fig. 213). When the above ratio was diluted 4–8 times, the antigenic activity, as detected by the number and strength of precipitin lines, fell sharply. At twice the dilution very little difference in the number and strength of precipitin lines was detected, and when the extractions were made up of even higher concentrations of powder, no detectable increase in the number or strength of lines was noted (Fig. 213).

Antigens were used, either as whole-powder suspensions, or as particle-free supernatants from centrifugations of the whole-powder suspension. Very little difference in antigenic activity (as described above) could be detected between the whole-powder suspensions and the supernatant extracts, except possibly by a bit weaker activity in the latter (Fig. 213). Even for immunoelectrophoresis, the whole-powder suspension was quite satisfactory.

3. Protein determinations

Three different methods of protein determination were used at one time or another in the course of this investigation. They were: (1) Micro-Kjeldahl (Burris and Wilson, 1957); (2) Biuret (Gornall, Bardawill, and David, 1949); and (3)

TABLE 18. *Soluble protein concentration of Chlorococcum and Tetracystis species used for immunochemical investigations (Folin-phenol method of Lowry; Crystalline B.S.A., Armour, used as a standard)*

Chlorococcum	Protein concentration mg/ml	*Tetracystis*	Protein concentration mg/ml
C. diplobionticum	16.3	*T. aeria* (C—6)	11.0
C. echinozygotum	9.5	*T. aeria* (Pa—3)	14.3
C. ellipsoideum	16.3	*T. aggregata*	17.0
C. hypnosporum	11.0	*T. aplanosporum*	6.5
C. macrostigmatum	11.0	*T. dissociata*	13.0
C. minutum	11.0	*T. excentrica*	12.5
C. multinucleatum	12.5	*T. illinoisensis*	15.0
C. oleofaciens	15.0	*T. intermedium*	9.0
C. perforatum	10.0	*T. isobilateralis*	11.8
C. pinguideum	10.5	*T. pampae*	11.0
C. punctatum	13.0	*T. pulchra*	14.3
C. scabellum	19.0	*T. tetrasporum*	11.0
C. sp. (tetra isolate)	16.3	*T. texensis*	11.8
C. vacuolatum	2.3		
C. wimmeri	12.5		

Folin-phenol (Lowry *et al.*, 1951). Of these, the Folin-phenol method provided the most reliable data. Table 18 lists the species of *Chlorococcum* and *Tetracystis* investigated and the protein concentrations of their extracts. Bovine serum albumin was used as a standard. With the exception of *Chlorococcum vacuolatum*, the range in protein concentration varied from 6.5 mg/ml, in *T. aplanosporum*, to 19.0 mg/ml, for *C. scabellum*. Since one of the most potent antisera was obtained from *T. aplanosporum*, it appears that the proteins of 11 other species, with the possible exception of *C. vacuolatum*, were of sufficient concentration for effective immunization and testing. In addition, 2 species, namely *C. diplobionticum* and *C. echinozygotum*, differed by their protein concentration almost 2-fold, yet such a difference was hardly, if at all, detectable on the double-diffusion precipitin tests (Fig. 221). Figure 213 shows an experiment designed to test the useful range of antigen concentration of *C.* sp. (tetra) for double diffusion and immunoelectrophoresis. Note that sufficient reaction occurs with well Nos. 1 (32.6 mg protein/ml), 2 (16.3 mg protein/ml), and 3 (8.15 mg protein/ml). Detectable decreases in reactivity are noted at the protein concentrations of 4.7 mg protein/ml (well No. 4) and 2.3 mg protein/ml (well No. 5).

4. Preparation of antisera

Three different lots of rabbits were used for antiserum production. As techniques were perfected, the antisera from each succeeding lot of rabbits were improved. Therefore, techniques involving the first 2 lots of rabbits will be outlined only briefly.

The first lot of rabbits was injected with 2–10% saline suspension of whole cells grown in liquid culture (BBM). The resulting antisera were very poor in precipitating antibodies, even though agglutination titers as high as 1:8192 were apparently recorded.

The second batch of rabbits was injected with acetone-powder suspension and with ultrasonic fractionates (including fat-soluble materials). From this batch, it was concluded that the acetone-powder suspension induced a superior antiserum to that of the sonicated material. Of the acetone-powder antisera, only one was satisfactory for use in the final phase of this study, namely, the antiserum to *T. aeria* (C–6).

A third lot of rabbits was immunized with preparations which were higher in protein content, due to improvement in culture techniques by increasing the nitrogen content of the culture medium (see page 9), and also to improvement in the grinding techniques in the acetone-powder method. In addition, the writers were in a better position to choose more carefully those species which would be of most value.[1]

[1] However, it was later found that antisera to certain other additional species also would have been valuable.

Female New Zealand white rabbits were injected with preparations of 2 species of *Tetracystis* and 2 species of *Chlorococcum*. Control bleedings prior to immunization revealed no antibodies to algae. The routes of injections as well as the amounts of material injected were varied because of difficulties in obtaining sufficient antigen preparations for a complete injection series, and because antibody production did not always appear to proceed as expected. The frequent titer checks were made by double-diffusion against liquid extracts which had been frozen and thawed many times. Apparently poor reactions may have been due to the deteriorated extracts. Since the schedule was prolonged and complicated, the final production of potent antisera cannot be attributed to any 1 course of injections. The total series of injections is shown in Table 19.

TABLE 19. *Injection and bleeding schedule used in the production of antisera against Chlorococcum and Tetracystis species*

Injection Date (after time 0)[a]	Amount (in ml)	Route[b]
1,7,14	2 × 1.0	IM
30	1.0	IP
32,34,36,39,41,43	0.5	IV
58	4 × 0.1	TP
69	2 × 0.2	IM
76	1.4	SC
98	2 × 0.5	IM
99,101	0.15	IP
103,104	0.33	IP
107,110	0.5	IP
119,120,122,124	0.2	IV
126	2.5	IM

[a] Test bleeding dates: 12, 20, 28, 35, 40, 48, 86, 99, 103, 106, 120, 124, 131.
Mass-bleeding dates: 112, 133.

[b] Key to immunization route: IM = intramuscular, with Complete Freund's Adjuvant or with Sodium Alginate Adjuvant (COLAB); IP = intraperitoneal; IV = intravenous; SC = subcutaneous; TP = toe pad, with Complete Freund's Adjuvant; these are effective but very painful to the rabbits (Leskowitz, 1960).

With reference to algae, it is worthwhile to discuss briefly some of the probable essential points for the production of good anti-serum. These appear to be: (1) a large supply of acetone powder; (2) freshly prepared acetone-powder suspensions (soluble protein content about 2.0%); (3) the use of Complete Freund's Adjuvant; and (4) sufficient time for antibody production. A suitable course of injections might be: days 1 and 14 intramuscular, 1 ml emulsion with Complete Freund's Adjuvant into 2 sites; day 21, subcutaneous, 0.25 ml emulsion into 4 sites; days 35–49, on 5 days, intraperitoneal injections of 1.0 ml of suspension; or on 10

days, several intradermal injections of 0.2 ml in many sites. About day 55, bleed 40–60 ml of blood, if the titer is high. Titer checks should be made twice a week. After resting the rabbit for 3–4 weeks, this schedule may be repeated. Further information concerning injection schedules in general is given by Leskowitz (1960) and by Crowle (1961).

5. Processing of antisera

The fresh blood was collected in 40-ml centrifuge tubes and kept at room temperature for 1–2 hr to allow clotting, and then maintained for several more hours at 5° C. The serum was then decanted into another centrifuge tube and centrifuged to clear the solution. One drop of 20% sodium azide was added for every 5 ml of serum which was stored at −10° C until needed. Repeated freezing and thawing of the antiserum did not appear to affect its potency, nor did prolonged storage (up to 1 year).

6. Absorption of antisera

For unequivocably demonstrating antigen specificity of only 1 or 2 groups of species having several antigens in common, it was necessary to remove the common antibodies by absorption. Complete absorption could be obtained by adding 0.5 ml of antiserum to 0.5 ml of antigen of species of the second group (usually at a concentration of 11–14 mg protein/ml. This mixture was maintained at room temperature for 2 hr during the absorption process. The absorbed antiserum was cleared by centrifugation and reacted immediately on double-diffusion plates (Fig. 225–228).

7. Preparation of materials for double-diffusion and immunoelectrophoresis (general reference: Crowle, 1961)

(1) Preparation of Buffered Agar

Ionagar No. 2 (Oxoid, London, distributed by Consolidated Laboratories, Chicago) was employed at a final concentration of 0.6% when mixed with the buffer. A barbital buffer slightly modified from that given by Crowle (1961) was used in conjunction with the Ionagar. The buffered agar is made in 2 parts:

A. Agar (concentrate)

Ionagar	6.0 g
Deionized H_2O	500.0 ml

B. Barbital buffer (concentrate), ionicity approximately 0.05, pH 8.2

Sodium barbital	15.85 g
Deionized H_2O	310.0 ml
Hydrochloric acid, 0.1 N	190.0 ml

Both parts A and B were compounded separately the day before they were to be combined for pouring as separate aliquots. Part A was allowed to soak for 10–30 min before autoclaving for 15 min. Then it was allowed to cool and kept overnight to "mature." Part B was kept overnight to allow maximum electrolytic dissociation.

The next day, both A and B were heated separately in the autoclave, and then heated together for 1/2 hr at about 90° C. The hot, buffered agar solution was filtered twice through Whatman No. 4 paper in a hot Buchner funnel and dispensed in 16.5-ml aliquots into sterile, screw-capped glass vials, with a Brewer automatic pipetting machine. The agar was allowed to cool and set, and then 2 drops of 20% sodium azide were added. The caps were tightened and the agar was stored at room temperature until preparation of the plates.

(2) Preparation of glass plates

Lantern-slide cover glasses ($3\frac{1}{4}'' \times 4'' \times 1/16''$[1]) were cleaned with a silk cloth and then sprayed with 0.1% agar and dried 3 times so as to form a surface to which the agar would stick. The buffered agar aliquot was heated for about 10 min over a steam cone to melt the agar. The molten agar was carefully poured onto a perfectly leveled glass plate and allowed to solidify in a moist, dust-free atmosphere. The solidified agar plate was kept overnight to "mature" before use.

The next day, it was placed over a pattern drawn on paper, and the pattern was duplicated on the agar plate with the aid of a cork borer (4 mm), scapel and ruler. Trough widths ranged from 2 mm for immunoelectrophoresis to 4 mm for double-diffusion. All cup diameters were 4.0 mm. Cup-trough separation ranged from 0.4 to 0.5 cm. The cups and troughs were cleaned by means of a Pasteur pipette connected to a vacuum filter pump.

(3) "Running" the plate, double-diffusion

The antigen extracts were pipetted into the cups and the antisera into the troughs (or vice versa), and the plate was allowed to react (develop) for 24–28 hr in a moist, dust-free environment.

(4) "Running" the plate, immunoelectrophoresis

The antigen extracts were pipetted into the cups (the agar had been removed only from the cups and not the troughs at this time). A reference spot of bromophenol blue was injected into the agar plate which was then placed in the electrophoresis apparatus.[2] The plates were run at 45 milliamperes per plate (ca. 4 volts per cm in the agar). Suitable separation of the antigens was achieved by the time

[1] Burke and James, Inc., Chicago, Ill.

[2] Shandon Universal Electrophoresis Apparatus, distributed by Consolidated Laboratories, with a Beckman Model RD-2 Duostat regulated power supply.

the bromophenol blue reference spot moved from 4.0 cm (about 1.5 cm/hr). After electrophoresis, the troughs were removed by suction, and the antiserum was added. The plates were incubated overnight in a moist, dust-free atmosphere.

(5) Examination of the plates

The plates were examined 24–48 hr after adding the antigen and antiserum. Precipitin lines were generally at optimum formation and resolution after 24–36 hr. The plates were photographed by positioning over a fluorescent X-ray viewing light with a black background arranged so that the precipitin lines were visible to the camera. The plates were also washed, dried, and stained with 0.2% Ponceau S (for proteins) and preserved for further analysis (Crowle, 1961).

C. RESULTS AND DISCUSSION

1. Analysis of double-diffusion

Fifteen *Chlorococcum* species and 12 species of *Tetracystis* were reacted in double-diffusion experiments with 5 antisera. These antisera were: (1) anti-*T. aeria* (C–6 isolate); (2) anti-*T. isobilateralis*; (3) anti-*T. aplanosporum*; (4) anti-*C.* sp. (tetra isolate); and (5) anti-*C. perforatum.*

The double-diffusion plates were allowed to develop for at least 48 hr, after which they were washed, dried, and preserved. During development, they were photographed at 24, 36, and 48 hr. Thus, the analyses were based on continuous observation over a prolonged period because occasionally, several additional precipitin lines would develop. A comparison of the 24-hr (Fig. 217–220) and 48-hr (Fig. 221–224) plates of *Chlorococcum* species reveals such changes.

Table 20 presents data obtained by analysis of the double-diffusion spectra. The results with antisera to *Tetracystis* form 3 vertical columns to the left and those with antisera to *Chlorococcum* form 2 vertical columns to the right. The horizontal columns represent the number of precipitin lines observed, ranging from 2 to 7. The various *Chlorococcum* and *Tetracystis* species are arranged on the basis of the number of precipitin bands they produced with each respective antiserum. Photographs of the double-diffusion tests with *Chlorococcum* and *Tetracystis* species are shown in Fig. 214–224.

Table 20 also is coded so that analyses are easier to follow. All presently designated *Chlorococcum* species are in bold face type, all *Tetracystis* species Roman type. Those species with an asterisk (including both *Chlorococcum* and *Tetracystis* species) produced full spectra with more than 5 lines with at least 1 antiserum.

The fullest spectra produced by any *Tetracystis* species were with anti-*Tetracystis* antisera. These same species gave poor reactions (3–4 lines or less) with the anti-*Chlorococcum* antisera. Conversely, the fullest spectra produced by any *Chlorococcum* species were with anti-*Chlorococcum* antisera. Again, these species gave poor reactions (3–4 lines or less) with the anti-*Tetracystis* antisera.

TABLE 20. *Double-diffusion reactions of Tetracystis and Chlorococcum*

T. aplano.	T. aeria (C-6)	T. iso.	C. sp. (tetra)	C. perf.	No. of lines
T. aplano.*					7
	T. aeria* (C-6) T. aeria* (Pa-3)	T. dissoc.* T. iso.* T. illin.* T. aggreg.*	**C. sp.*** **C. scab.*** **C. oleo.***		6–7
		T. aeria* (C-6)		**C. perf.*** **C. echino.*** **C. diplo.***	6 5–6
	T. aggreg.* **C. hypno.** T. pam.	T. aeria* **C. wim.** (Pa-3)	**C. multi.**		5
T. aggreg.* **C. oleo.** **C. punct.** **C. multi.**	T. dissoc.* T. illin.* T. iso.*	**C. ellip.** **C. min.** **C. punct.** **C. multi.**	**C. perf.*** **C. ellip.** **C. min.** **C. vacuo.**	**C. wim.** **C. multi.**	4–5
T. dissoc.* **C. ellip.** T. illin.* **C. min.** T. iso.* **C. wim.** T. tetra. T. excent. T. texen. T. pulch. T. inter.	T. excent. **C. min.** **C. ping.**		T. excent. **C. echino.*** **C. diplo.*** **C. wim.** **C. punct.** **C. ping.**	**C. oleo.*** **C. ellip.** **C. punct.** **C. vacuo.**	4

T. aeria* (C-6) T. aeria* (Pa-3) T. pam.	**C. hypno.** **C. scab.*** **C. ping.** **C. macro.**	T. tetra. T. inter.	**C. perf.*** **C. echino.*** **C. diplo.*** **C. ellip.** **C. wim.**		**C. hypno.** **C. oleo.*** **C. ping.** **C. vacuo.** **C. macro.**	T. aeria* (C-6) T. aeria* (Pa-3) T. texen. T. pulch.	**C. macro.**		**C. scab.*** **C. min.** **C. ping.**	3–4
	C. sp.* **C. perf.*** **C. echino.** **C. diplo.*** **C. vacuo.**	T. texen. T. pulch.	**C. sp.*** **C. scab.*** **C. oleo.***	T. excent.	**C. echino.***	T. aggreg.* T. inter. T. pam.		T. aggreg.* T. excent. T. texen.	**C. sp.*** **C. macro.**	3
			C. punct. **C. multi.** **C. macro.**	T. aplano.* T. tetra. T. pulch. T. inter.	**C. sp.*** **C. scab.*** **C. perf.*** **C. diplo.***	T. dissoc.* T. illin.* T. iso.* T. tetra.	**C. hypno.**	T. aeria* (C-6) T. dissoc.* T. tetra. T. pulch. T. inter.	**C. hypno.**	2–3
		T. aplano.*	**C. vacuo.**	T. pam. T. texen.		T. aplano.*		T. aplano.* T. illin.* T. iso.* T. aeria* (Pa-3) T. pam.		2 or less

* Species which produced full spectra with more than 5 lines with at least 1 antiserum.

These results indicate inter-generic serological specificity inasmuch as, in general, any species producing a full spectrum (over 5 lines) did so with an antiserum to a species in the same genus. The same species produced poor spectra (less than 3–4 lines) with all antisera to species in the other genus. However, in many cases such species only gave medium spectra (4, 4–5, or 5 lines) with antisera to other species in the same genus. This would indicate specificity not only for the whole genus, but in particular, for individual groups of species within the genus.

In addition to an analysis of just the fully reacting species, all species of *Tetracystis* and *Chlorococcum* were examined for possible intergeneric or group specificity. For each antiserum, the number of species in either genus producing full and medium reactions (over 3–4 lines) was compared with the number of species giving poor reactions (3–4 lines or less). Reciprocal comparisons were made in each case for the *Tetracystis* species (T) and the *Chlorococcum* species (C). The results obtained are shown in Table 21.

TABLE 21. *Reciprocal comparisons for Chlorococcum and Tetracystis species in double-diffusion*

Antiserum	*T. aplanosporum*		*T. aeria*		*T. isobilateralis*	
Antigen, genus	T	C	T	C	T	C
over 3–4 lines	10	6	8	3	6	5
3–4 lines or less	3	9	5	12	7	10

Antiserum	*C. sp. (tetra)*		*C. perforatum*	
Antigen, genus	T	C	T	C
over 3–4 lines	1	13	0	9
3–4 lines or less	12	2	13	6

In Table 21, the abbreviation above each fraction indicates the species of *Chlorococcum* (C) or *Tetracystis* (T) reacted against the antiserum listed. The "numerator" in all cases, represents the number of species which fell above the division line of 3–4 precipitin lines, while the "denominator" indicates those species falling below the division line in Table 20. Note that in all cases, a total of 13 *Tetracystis* species and 15 *Chlorococcum* species were tested. It can be seen, first, that a reciprocal relationship exists in the reactions of the 2 genera against a given antiserum. Secondly, the pair of fractions for any given antiserum of *Tetracystis* is the reverse of that for any *Chlorococcum* antiserum. Thus, when all of the species are considered, they indicate intergeneric specificity in almost every combination tested as shown in Table 21.

Chlorococcum hypnosporum gave unexpected and very interesting reactions (Table 20) in that it reacted weakly with the 2 *Chlorococcum* antisera but quite

strongly (5 precipitin lines) against one of the *Tetracystis* antisera, namely, anti-*T. aeria*. This reaction may not be considered too suspect because the hypnospores which characterize this *Chlorococcum* species may, in fact, represent nonmotile daughter vegetative cells and not aplanospores. Therefore, in view of the writers' concepts for the Chlorosphaerales (page 11), a re-examination of *C. hypnosporum*, using an antiserum against it would provide even more conclusive data with regard to its taxonomic position.

From Table 20, it is clear that *T. pampae* is certainly unusual serologically because of the poor reactivity with all of the *Chlorococcum* and 2 of the *Tetracystis* antisera. An exception is the 5-line precipitin reactions with the antiserum to *T. aeria*. This reaction, however, is weak when compared with the 5-line precipitin reactions given by the other 2 species against *T. aeria* antiserum. The protein concentrations of the species giving 5-line reactions against *T. aeria* are the same in 2 of these species, including *T. pampae* (Table 18). Therefore, the difference in intensity strength of reaction probably indicates that *T. pampae* is not so similar serologically to the other 2 species.

Thus it may be proper at this point to state that serological similarity or diversity cannot be based only on the number of precipitin lines produced in double-diffusion as in Table 20. The number of lines alone (Table 20) does not indicate antigens which are specific and which are in common to other species; however, this may be shown in the photographs of the double-diffusion tests (Fig. 214–224). In these photographs, the presence of spurs or arcs denotes those antigens which are not in common with adjacent species. A difference of 1 or 2 in the number of precipitin lines may not be very significant, whereas a spur indicates a significant difference. For this reason, techniques with absorption and immunoelectrophoresis were employed in order to display more fully these reactions and to understand their significance. It should be emphasized, however, that the number of precipitin lines (Table 20) does provide valuable information and should be given full attention in a first approach to the various modes of serological analysis.

The antisera to *T. aeria* and *T. isobilateralis* appear to show differences among those *Tetracystis* species which undergo intervening diad formation and those which form tetrads directly. It will be recalled that *T. isobilateralis* is quite distinctive in forming diads which divide to form isobilateral or tetrahedral tetrads. Other species of *Tetracystis* do not have this same capability in that they appear exclusively to form tetrads directly. Note in Table 20 that those *Tetracystis* species which undergo intervening diad formation react most strongly (6–7 lines) with anti-*T. isobilateralis*. Note also that these species do not react so strongly (4–5 lines) with antiserum to *T. aeria* which forms tetrads directly. It is apparent that all species of *Tetracystis* which characteristically form tetrads directly react more strongly to anti-*T. aeria* than to anti-*T. isobilateralis*.

The fully reacting species will now be discussed because, on the basis of double-diffusion, several species-groups could be discerned. When *Tetracystis aeria* isolate

C–6 was reacted against its homologous antiserum, a very strong reaction of 6–7 lines was detected (Fig. 214, Table 20). Likewise, another isolate Pa–3, from a different locality, was tested against anti-*T. aeria* (C–6) and produced an identical spectrum to the homologous reaction (C–6). It will be recalled that these 2 isolates have been demonstrated to be the same species on the basis of their morphology, ultrastructure, and physiology. Thus, serological evidence also supports all these data.

The strongest reaction with anti-*T. aplanosporum* antiserum was produced by the homologous extract from *T. aplanosporum*. This reaction (7 lines) was unique in that no other *Tetracystis* species even closely approached it in strength (Fig. 215, Table 20). Thus, *T. aplanosporum* is immediately segregated as a unique species of *Tetracystis*. On the basis of morphology, *T. aplanosporum* and *T. aeria* are the only 2 presently known species of *Tetracystis* in which the nucleus of the zoospore is anterior. Morphological, physiological, and ultrastructural evidence show that the 2 species are only distantly related, even though they share the same zoospore nuclear position. These 2 species are serologically different from one another as is shown in Fig. 214–215 and Table 20.

Tetracystis isobilateralis, when reacted against its homologous antiserum, gave a very strong reaction of 6–7 lines in double-diffusion. Likewise, *T. illinoisensis*, *T. dissociata*, and *T. aggregata* gave equally strong and identical reactions with anti-*T. isobilateralis* antiserum. That these 4 taxa are not morphologically, physiologically, and ultrastructurally identical suggests that they are distinct species; however, they do have many similarities in common on the basis of morphology and ultrastructure. Thus, the serological data seem to support these similarities, while one is unable to distinguish among the 4 species on the basis of double-diffusion (Fig. 214–215, Table 20).

Among the *Chlorococcum* species, 2 serological groupings of importance were detected by double-diffusion. The first group, which reacts strongly and similarly with anti-*C.* sp. (tetra) antiserum, consisted of *C.* sp. (tetra), *C. scabellum*, and *C. oleofaciens* (Fig. 216, 219, 220; Table 20).

The second group reacted strongly with anti-*C. perforatum* antiserum and consisted of *C. perforatum*, *C. echinozygotum*, and *C. diplobionticum*. These reactions appeared to be identical on the basis of double-diffusion (Fig. 217, 221; Table 20).

2. Absorption studies

The writers were limited by shortages of time and material in performing absorption experiments. However, enough material was available to study more critically 2 serological groups among *Tetracystis*, namely, the *T. aeria* (C–6 and Pa–3) group, and the *T. isobilateralis* group (consisting additionally of *T. dissociata*, *T. illinoisensis*, and *T. aggregata*). Figures 225 and 226 show reactions of these 2 species groupings in which the antisera to *T. isobilateralis* and *T. aeria* (C–6) were cross-absorbed reciprocally. The controls (non-absorbed antisera)

were also included. Anti-*T. isobilateralis* absorbed with *T. aeria* (Pa-3) produced no reaction against *T. aeria*, suggesting complete absorption. Likewise, anti-*T. aeria* absorbed with *T. isobilateralis* reproduced no reaction against *T. isobilateralis*.

Anti-*T. isobilateralis* antiserum absorbed with *T. aeria* (Pa–3) produced no reaction with either Pa–3 or C–6 isolates of *T. aeria*, thus indicating that isolate C–6 has no more antigens in common with *T. isobilateralis* than does isolate Pa–3. Anti-*T. aeria* antiserum absorbed with *T. isobilateralis* produced no reaction to *T. aggregata*, *T. dissociata*, and *T. illinoisensis*, likewise indicating that these 3 species have no more antigens in common with *T. aeria* than does *T. isobilateralis*.

Extracts of *T. isobilateralis*, *T. aggregata*, *T. dissociata*, and *T. illinoisensis* reacted with anti-*T. isobilateralis* antiserum absorbed with *T. aeria* all produced identical spectra of 2 lines, indicating complete antigenic uniformity of these 4 species. Likewise, with anti-*T. aeria* antiserum both isolates, C–6 and Pa–3 produced identical spectra of 4 lines, indicating antigenic identity of these 2 species.

Chlorococcum wimmeri, which had appeared similar to both *Tetracystis* groups in double-diffusion tests, was here shown to be very different from both of them in that with either absorbed antisera, it produced no reaction (Fig. 225–226).

A second absorption experiment was performed in which antisera to *T. aeria* and *C.* sp. (tetra isolate) were cross-absorbed reciprocally. *Chlorococcum* and *Tetracystis* species selected to be most representative for each genus were reacted against the absorbed antisera (Fig. 217, 218, Table 22). There was evidence for incomplete absorption with one of the antisera, because anti-*C.* sp. absorbed with *T. aeria* (Pa–3) gave a single faint precipitin line when reacted against *T. aeria* (Pa–3). For this reason, the significant reactions to absorbed anti-*C.* sp. must be considered 1-precipitin-band less than the observed number.

Anti-*T. aeria* absorbed with *C.* sp. was completely absorbed; therefore, every precipitin line observed against this absorbed antiserum was considered significant. From this absorption experiment, it is evident that almost all of the *Tetracystis* species appear to have at least 1 genus-specific antigen to anti-*T. aeria* (absorbed with *C.* sp.). *Tetracystis pampae* is the exception. Most of the *Chlorococcum* species tested lacked specific antigens to the absorbed *Tetracystis* antiserum. The exceptions were *C. hypnosporum* and, possibly, *C. ellipsoideum.*

Conversely, against absorbed antiserum *C.* sp. all the *Chlorococcum* species tested (with the exception of *C. hypnosporum* and *C. ellipsoideum*) have at least 1 genus-specific antigen. All of the *Tetracystis* species tested lacked this antigen, assuming that anti-*C.* sp. was incompletely absorbed by 1 precipitin band.

Therefore, the absorption data from this experiment seem to indicate an intergeneric specificity with both fully and poorly reacting *Chlorococcum* and *Tetracystis* species. Even though not all of the *Tetracystis* species are represented, they nevertheless represent the complete antigenic diversity among all species. Those species of *Tetracystis* not reacted, belong to *T. aeria* group and the *T. isobilateralis group*, each of which was shown to be serologically identical in the first absorption

TABLE 22. *Data from the second absorption experiment with selected Chlorococcum and Tetracystis species*

Species (antigen)	Reaction with anti-*T. aeria* (-*C.* sp)		Reaction with anti-*C.* sp. (-*T. aeria*)	
	No. of lines	Quality of spectrum	No. of lines[a]	Quality of spectrum
T. aeria (Pa-3)	4	very strong	0	—
T. isobilateralis	3	weak	0	—
C. hypnosporum	3–4	weak	0[b]	—
T. texensis	ca. 2	weak	0	—
T. excentrica	1	weak	0	—
T. intermedium	1	weak	0	—
T. aplanosporum	1	weak	0	—
T. pampae	0	—	0[b]	—
C. ellipsoideum	1	very weak	0	—
C. perforatum	0	—	1	weak
C. echinozygotum	0	—	1	weak
C. diplobionticum	0	—	1	weak
C. pinguideum	0	—	1	weak
C. sp. (tetra)	0	—	4–5	very strong

[a] This number represents 1 minus the observed number = corrected number of incomplete absorption.

[b] No lines were present, even before compensation for incomplete absorption.

study. In the case of the *Chlorococcum* species, this absorption was not entirely representative of every species of the genus, as in *Tetracystis*.

Most of the species tested in the second absorption experiment produced a spectrum of only 1 specific antigen; however, several species produced more than 1 specific antigen. Of these, *T. aeria* (Pa–3) had 4 specific antigens, against anti-*T. aeria* (-C. sp.), thus forming a species group by itself with the other isolate of *T. aeria* (C–6). *Tetracystis isobilateralis* produced a spectrum of 3 specific lines against anti-*T. aeria* (-C. sp.), thus forming a species group of *T. isobilateralis, T. dissociata, T. illinoisensis,* and *T. aggregata.* Against anti-*C.* sp. (-*T. aeria*), only *C.* sp. formed 5 specific antigens. This reaction represents probably the other 2 closely allied species, *C. oleofaciens* and *C. scabellum.*

Against anti-*T. aeria* absorbed with *C.* sp., *Chlorococcum hypnosporum* produced 3–4 specific lines, while against anti-*C.* sp. absorbed with *T. aeria,* no lines were produced. These data further support the double-diffusion experiments which indicated that *C. hypnosporum* has more antigens specific to *Tetracystis* than to *Chlorococcum.* The absorption data also show that *C. hypnosporum* is especially close to the *T. aeria* and *T. isobilateralis* groups. Of these groups, *C. hypnosporum* shares approximately the same number of specific antigens with *T. isobilateralis* (3–4), while *T. aeria* has at least 1 additional specific antigen, all of which are stronger in intensity than those of *C. hypnosporum* and *T. isobilateralis.* It could

not be stated with certainty whether the specific antigens shared by *C. hypnosporum* and *T. isobilateralis* were exactly the same, because these 2 species were not placed side-by-side in the agar.

Tetracystis pampae has no specific antigens to either the *Tetracystis* or *Chlorococcum* absorebd antisera. Thus, it is only distantly related to *T. aeria* and *C.* sp. Further studies with *T. pampae* antiserum would certainly be of great value in determining if this species is as unique serologically as it is in its morphological, physiological, and ultrastructural characters.

It is of interest to note that *Chlorococcum ellipsoideum* shows a single, weak line with the absorbed *Tetracystis* antiserum and with the incompletely absorbed *Chlorococcum* antiserum, only 1 line, the latter not significant. *Chlorococcum ellipsoideum*, then, appears to be related to *Tetracystis* in a similar, but less striking, manner than *Chlorococcum hypnosporum*. Further work will be needed in order to test this possibility.

Although far from complete, these few simple investigations have shown the value of this technique in conjunction with double-diffusion tests. The value of serology is increased yet further with the aid of immunoelectrophoresis which will now be discussed.

3. Immunoelectrophoresis analysis (I.E.A.)

Twenty-four preparations of *Tetracystis* and *Chlorococcum* species were analyzed by immunoelectrophoresis with 3 antisera to *Tetracystis* and 2 antisera to *Chlorococcum*. These reactions are summarized in Table 23. Immunoelectrophoresis was performed in order to aid in the analysis of double-diffusion and absorption data. Immunoelectrophoresis analysis (or I.E.A. as it is commonly referred to) has the distinct advantage of resolving many more precipitin bands (Table 23) than could be observed by double-diffusion (Table 20). In addition, electrophoresis mobilities of specific proteins may be resolved with this technique. Thus apparent identity of spectra in double-diffusion may be proven or disproven by I.E.A.

Six major species groupings discerned with double-diffusion were more critically analyzed by I.E.A., as were certain other species which were of interest. Reactions with each species group will now be discussed.

(1) The *T. aplanosporum* group (Fig. 233, 234)

It will be recalled that in double-diffusion, the strongest reaction to anti-*T. aplanosporum* was produced by the homologous extract from *T. aplanosporum* (7 lines). With I.E.A., 1–3 additional precipitin lines (over that of double-diffusion) were detected with the homologous reaction. In I.E.A. against anti-*T. aplanosporum* antiserum, *T. aplanosporum* was unique in having more lines than any other species, and these were stronger and had different electrophoretic mobilities.

TABLE 23. *Immunoelectrophoresis reaction performed with number of lines indicated*

Antisera	*T. aplanosporum*	*T. aeria*	*T. isobilateralis*	*C.* sp. (*tetra*)	*C. perforatum*
T. aplanosporum	8–10	....	....	....	....
T. aeria (C-6)	5–6	11	5–6	....	....
T. aeria (Pa-3)	....	11	5–6	....	....
T. isobilateralis	....	8–9	8–9	....	....
T. aggregata	6–7	8–9	7–8	....	....
T. illinoisensis	5 or 6	8–9	7–8	....	....
T. dissociata	6–7	8–9	7–8	....	....
C. hypnosporum	6–7	8	....	....	....
C. wimmerii	6–7	....	5–6	4	5–6
C. sp. (tetra)	....	3	....	10–12	....
C. scabellum	....	....	....	9–12	....
C. oleofaciens	5–6	....	....	9 or 10	....
C. multinucleatum	....	....	....	8–9	6
C. ellipsoideum	5 or 6	4–5	5	7	6–7
C. perforatum	....	....	....	6 or 7	8–10
C. diplobionticum	....	....	....	6 or 7	9–10
C. echinozygotum	....	....	....	4	9
C. minutum	4	3–4	3	3–4	2 or 3
C. vacuolatum	....	....	....	7	5
C. punctatum	....	....	....	5 or 6	6
C. pinguidium	....	3–4	....	....	....
T. pampae	....	2 or 3	....	....	....
T. excentrica	....	4	3–4	....	....
T. intermedium	....	4–5	3–4	....	....
T. pulchra	....	4	4	....	....

(2) The *T. isobilateralis* group (Fig. 229–232)

Tetracystis aggregata, T. dissociata, T. illinoisensis, and *T. isobilateralis* gave very similar, if not identical, reactions by double-diffusion. Therefore, it was of interest to examine them by I.E.A. in order to determine if they were, indeed, identical serologically. Against antisera to *T. aplanosporum* and *T. aeria*, the reactions to all species comprising this group were very similar, if not identical, in number, intensity, and electrophoretic mobility. Only *T. isobilateralis* produced 1 extra precipitin band when reacted with anti-*T. isobilateralis*. Thus, all members comprising the *T. isobilateralis* group appear to be serologically virtually identical on the basis of double-diffusion, absorption, and immunoelectrophoresis.

(3) The *T. aeria* group (Fig. 229–232)

According to the double-diffusion and absorption data, both isolates C–6 and Pa–3 of *T. aeria* are serologically identical. The same holds true when these 2 iso-

lates were tested by I.E.A. Note that against anti-*T. aeria* (C–6) both isolates produced identical spectra of 11 lines (Fig. 229), while against anti-*T. isobilateralis* they both produced identical spectra of 5–6 lines (Fig. 230). It is of interest at this point to note that most of the C–6 and Pa–3 specific proteins (not present in the *T. isobilateralis* group) formed arcs in the general area of the origin (near the cups). Thus, they were proteins of low electrophoretic mobility, either because of a small net charge or large molecular size. It can be deduced, therefore, that there is greater specificity in the proteins of larger molecular size in this instance.

Note that in the cross reactions between the *T. aeria* group and the *T. isobilateralis* group, there were 2–4 lines less than between the homologous reactions, thus indicating that each group has 2–4 specific antigens not shared by the other group. This observation was verified with absorption and even more critically in that the *T. aeria* group shared 4 specific antigens, while the *T. isobilateralis* groups had 2 specific antigens.

(4) *C. hypnosporum* (Fig. 234, 240)

The double-diffusion and absorption studies indicated that *C. hypnosporum* was unique among the *Chlorococcum* species tested in that it more closely resembled species in *Tetracystis* than any in *Chlorococcum*. In the I.E.A. study, *C. hypnosporum* was reacted against anti-*T. aplanosporum* and anti-*T. aeria*. Against anti-*T. aplanosporum*, *C. hypnosporum* had a spectrum of similar strength and number of lines, to members of the *T. isobilateralis* group; however, not all of the precipitin lines of *C. hypnosporum* were of the same electrophoretic mobilities. *Chlorococcum hypnosporum* had several additional lines not represented by *T. aeria*, and in addition, lines with different electrophoretic mobilities were observed between *C. hypnosporum* and the latter.

Against anti-*T. aeria*, *C. hypnosporum* was outstanding in producing a strong spectrum with about 8 lines, similar in number and strength to those of the *T. isobilateralis* group (Fig. 240). However, there were obvious differences in the relative electrophoretic mobilities of many lines between the *T. isobilateralis* group and *C. hypnosporum*. From this evidence, it could be postulated that *C. hypnosporum* is in the general complex with the *T. aeria* and *T. isobilateralis* groups. However, on the basis of electrophoretic mobilities, *C. hypnosporum* is in a group separate from the *T. isobilateralis* group. It was unfortunate that the writers did not have the time nor material to explore the I.E.A. reactions of *C. hypnosporum* to anti-*T. isobilateralis*.

(5) The *C.* sp. (tetra) group (Fig. 237–239)

The reactions of *C.* sp. (tetra), *C. scabellum*, *C. oleofaciens*, and possibly also *C. multinucleatum* were quite similar in intensity and number of lines to anti-*C.* sp.

(tetra) on double-diffusion. For this reason they were investigated more critically by immunoelectrophoresis.

Against anti-*C.* sp. (tetra), *Chlorococcum scabellum* and *C.* sp. (tetra) produced similar numbers of lines with equal intensity and of the same electrophoretic mobilities (Fig. 237). However, *Chlorococcum oleofaciens* lacked 2 or 3 lines present to *C.* sp. (tetra). *Chlorococcum multinucleatum* had almost as full a spectrum as *C. oleofaciens* and was like it in the electrophoretic mobility of the precipitin lines present, and in the lack of 2–3 lines which were present with *C.* sp. (tetra) and *C. scabellum.*

Thus, all 4 species form a general complex, and within this complex, *C.* sp. (tetra) and *C. scabellum* appear to have identical reactions, while *C. oleofaciens* and *C. multinucleatum* (Fig. 238), although not identical, are both different from the *C.* sp. (tetra)-*C. scabellum* group.

(6) The *C. perforatum* group (Fig. 235–236)

Three species of *Chlorococcum*, namely, *C. perforatum*, *C. echinozygotum*, and *C. diplobionticum* gave almost indistinguishable reactions with double-diffusion; however, when examined with I.E.A., several differences among these species were detected. Against anti-*C. perforatum*, *Chlorococcum perforatum* and *C. echinozygotum* gave similar spectra in terms of number, intensity, and electrophoretic mobility. In contrast, some lines were absent with *C. diplobionticum* (Fig. 236), and some present had different electrophoretic mobilities.

All 3 species of the *C. perforatum* group produced different spectra against anti-*C.* sp. (tetra) antiserum.

Thus, in double-diffusion all 3 species of the *C. perforatum* group gave indistinguishable reactions, but I.E.A. showed that within this complex, *C. perforatum* and *C. echinozygotum* form a group of closely related species, while *C. diplobionticum* is more different.

(7) Miscellaneous reactions (Fig. 235–236; 238–240)

In addition to the species in the clear-cut groups displayed by double-diffusion, several other species were studied with I.E.A. in order to try to resolve their relationship to the better defined groups.

It will be recalled that *Chlorococcum ellipsoideum* gave between 3 and 5 precipitin bands with all antisera on double-diffusion. Against anti-*T. aplanosporum*, *C. ellipsoideum* produced 5 or 6 lines similar to those of *T. aeria* (C–6) (Fig. 233); however, they were of different electrophoretic mobility. Against anti-*T. aeria*, *C. ellipsoideum* had about 3 of the 5 total lines similar to *T. excentrica*, *T. intermedium*, and *T. pulchra*. Against anti-*T. isobilateralis*, *C. ellipsoideum* produced spectra more similar to *T. excentrica* than to *T. pulchra* or *T. intermedium*. There was no particular affinity of spectra with *C. minutum*, even though the number

of lines against this antiserum was about the same. Against anti-*C.* sp. (tetra), *C. ellipsoideum* produced more lines than against all other antisera, and showed a greater similarity to *C. scabellum* and *C. diplobionticum* than to other species tested. This relation should be regarded as rather weak, however, since there were several major line differences. *Chlorococcum ellipsoideum* reacted weakly with anti-*C. perforatum* but showed more similarly to *C. perforatum* and *C. echinozygotum* than any other species tested with this antiserum.

Therefore, in I.E.A. *Chlorococcum ellipsoideum* shows, as in double-diffusion, similarities to all of the antisera tested. Thus, this species may represent a link between the 2 genera. The absorption data seem to indicate that *C. ellipsoideum* may have, (in one case), a specific antigen to *Tetracystis* not present in *Chlorococcum* when reacted against absorbed *T. aeria* (-*C.* sp.). An antiserum to *C. ellipsoideum* would be, indeed, very useful, for no further conclusions can be made on the present basis. Likewise, other groups tested which gave very weak reactions in I.E.A. should be re-examined using an antiserum against one or more of these species, for no definite conclusions, other than those obtained by double-diffusion and absorption, could be discerned with I.E.A.

D. CONCLUSIONS

In conclusion, the writers were able successfully to immunize rabbits to 5 different species of algae representing 2 different genera, *Chlorococcum* of the Chlorococcales, and *Tetracystis* of the Chlorosphaerales. The reactions of 27 antigens suggested that the techniques of double-diffusion, absorption, and immunoelectrophoresis could be successfully employed to gain valuable knowledge regarding proper disposition of taxa at the species and generic level. There was some evidence that most members of each genus have at least 1 specific antigen which is lacking in the other genus, suggesting an intergeneric, serological specificity. Even though at least 1 genus-specific antigen may be present, there are at least 2–3 common antigens shared by most species of both genera. This is rather unique serologically in that *Tetracystis* and *Chlorococcum* presently are classified in 2 different orders. Such common antigenicity rarely, if ever, occurs at the ordinal level in the angiosperms. In view of this situation, it is possible that the present basis of ordinal distinction between the Chlorococcales and Chlorosphaerales may represent a more artificial classification than heretofore believed. On the other hand, the taxonomic significance of the antigens which both genera share in common may have been overestimated, because only recently, a common antigen (protein fraction 1) has been shown to be present among all major groups of the green plants, from the angiosperms to the Chlorophyta (Dorwer, Kahn, and Wildman, 1958).

Finally, the data from double-diffusion, absorption, and immunoelectrophoretic studies indicate that there are species groupings in these unicellular algae. Although these are probably natural assemblages, they would be useful, even if arti-

ficial, in the identification of species. The species groupings which are based on serological criteria, coincide, in general, with the groupings suggested by morphological (both light and electron-microscopic) and physiological attributes. This last consideration inspires some confidence that these morphological and physiological criteria of themselves, in fact, make it possible to classify these microalgae phylogenetically.

V. General Discussion

The data representing the morphology, physiology, and immunochemistry have been considered independently and are herein presented in summary form based on the assignment of the same letters for identical response to a given test. These data are summarized in Tables 24, 25 and 26.

TABLE 24. *Electron-microscopic data (based on the comparative study of organellar types)*

Ultrastructure	1[a]	2	3	4	5	6
Organism						
T. aeria (C-6)	C[b]	A	B	B	B	B
T. aeria (Pa-3)	C	A	B	B	B	B
T. aggregata	B	D	A	A	A	A
T. aplanosporum	C	C	D	D	D	C
T. dissociata	C	D	B	A	A	A
T. excentrica	C	B	C	C	C	C
T. illinoisensis	C	D	B	C	B	B
T. intermedium	C	D	C	C	C	C
T. isobilateralis	A	D	A	A	A	B
T. pampae	C	D	E	C	E	C
T. pulchra	C	B	C	C	C	C
T. tetrasporum	X[c]	X	X	X	X	X
T. texensis	C	D	C	C	C	C

[a] 1 = mitochondrion; 2 = cell wall; 3 = pyrenoid; 4 = chloroplast mass; 5 = chloroplast internal; 6 = Golgi apparatus.

[b] Letters A through E are based on the groupings in Table 17.

[c] X = no data.

Table 25 is based on the physiological data. For example, in the physiological test for growth in various carbon sources (Table 1), both isolates *T. aeria* fall into group 3 as shown in the species groupings (page 37). To these isolates and to other species of *Tetracystis* which fall into group 3 for this physiological test are ascribed the letter C in Table 25. Likewise, *T. pampae*, *T. aplanosporum*, and *T. intermedium*, fall into group No. 1 and thus have ascribed to them the letter A in Table 25.

In similar fashion, serological and ultrastructural data have been collated and are presented in Tables 24 and 26. Thus, when reading Tables 24–26 horizontally, the sequence of letters indicates the degree of relationship of the taxa. For example, it will be seen that both isolates of *T. aeria* have the same sequence of letters (CABBBB) on the basis of ultrastructural data, (CAAABAAAAA) for physiological data, and (DJHBD) for serological data. On this basis, a summation table has

TABLE 25. *Physiological data (based on species groupings)*

Physiological tests	1	2	3	4	5	6	7	8	9	10
Organism										
T. aeria (C-6)	C[a]	A	A	A	B	A	A	A	A	A
T. aeria (Pa-3)	C	S	S	S	B	A	A	A	A	A
T. aggregata	C	E	E	B	B	B	C	C	D	C
T. aplanosporum	A	B	B	C	C	B	C	A	C	D
T. dissociata	C	E	D	B	B	A	C	C	B	D
T. excentrica	C	E	D	B	D	S	C	B	C	C
T. illinoisensis	C	X	X	X	X	X	X	X	D	X
T. intermedium	A	E	F	C	D	B	B	A	C	D
T. isobilateralis	C	E	B	B	B	B	C	C	D	C
T. pampae	A	D	C	C	B	B	A	A	C	C
T. pulchra	C	X	X	X	X	X	X	X	A	X
T. tetrasporum	D	C	D	B	A	B	B	A	E	B
T. texensis	B	E	D	D	D	B	C	B	B	C

[a] Group numbers based on data from species groupings (pp. 37–40).
Group 1 = A
Group 2 = B
Group 3 = C
Group 4 = D
Group 5 = E
Group 6 = F
X = no data.

been prepared to indicate the over-all relationship of morphological, physiological, ultrastructural, and serological characteristics (Table 27).

In Table 27, species groupings indicating general relationship of species to one another are indicated by those species which fall within a given square for each category of study (i.e., morphology, electron microscopy, etc.). The degree of relationship to one another of the species within each square is indicated by various symbols placed after the specific names.

From this summary table, it is clear that the ultrastructural, physiological, and serological data correlate quite well with the morphological data. It should be emphasized that the morphological groups as presented here were derived independently, even before ultrastructural, physiological, and serological data had been obtained. It is of special interest to note the striking degree of identity of both isolates of *T. aeria* in all tests performed, this suggesting further that these 2 isolates are, indeed, the same species.

On the basis of morphology, *T. isobilateralis*, *T. dissociata*, *T. illinoisensis*, and *T. aggregata* form a group of closely related species; however, *T. illinoisensis* shows some degree of dissimilarity on the basis of ultrastructure and physiology, while *T. aggregata* lacks a very close relationship to the other members of this group on the

TABLE 26. *Serological data (based on the number of lines observed by double-diffusion)*

	Tetracystis			*Clorococcum*	
Antisera	1[a]	2	3	4	5
Organism					
T. aeria (C-6)	D[b]	J	H	D	B
T. aeria (Pa-3)	D	J	G	D	A
T. aggregata	F	G	J	C	C
T. aplanosporum	K	A	B	A	A
T. dissociata	E	F	J	B	B
T. excentrica	E	E	C	E	C
T. illinoisensis	E	F	J	B	A
T. intermedium	E	D	B	C	B
T. isobilateralis	E	F	J	B	B
T. pampae	D	G	A	C	A
T. pulchra	E	C	B	D	B
T. tetrasporum	E	D	B	B	B
T. texensis	E	C	A	D	C

[a] Antisera
1 = *T. aplanosporum*
2 = *T. aeria*
3 = *T. isobilateralis*
4 = *C. perforatum*
5 = *C.* sp. (tetra)

[b] Number of precipitin lines
A = 2 or less
B = 2–3
C = 3
D = 3–4
E = 4
F = 4–5
G = 5
H = 5–6
I = 6
J = 6–7
K = 7

basis of serology. Such variation is to be expected because the organisms in this group differ significantly in morphology, but not sufficiently to warrant a species description for each taxon.

Two species, namely, *T. aplanosporum* and *T. pampae*, are quite distinct from any other species of *Tetracystis* on the basis of morphology. They are also distinct on the basis of ultrastructure and serology; however, they show some degree of relationship to one another and to *T. intermedium* on the basis of physiology.

It is of interest to note that *T. tetrasporum*, although not unequivocally unique among the species of *Tetracystis* on the basis of morphology and serology, in fact, is so on the basis of physiology.

While *T. excentrica*, *T. texensis*, *T. pulchra*, and *T. intermedium* show some degree of morphological relationship to one another, it should be noted that *T. pulchra* and *T. intermedium* are very closely related morphologically, while *T. texensis* and *T. excentrica* are more distinct from the former 2 species. On the basis of ultrastructure, these 4 species cannot be differentiated. Serologically, *T. pulchra* and *T. texensis* are closely related, while *T. excentrica* is more closely related to *T.*

TABLE 27. *Summary: over-all relationship of Tetracystis species*

Morphology	Ultrastructure	Physiology	Serology
T. aeria (C-6) §	*T. aeria* (C-6) §	*T. aeria* (C-6) §	*T. aeria* (C-6) §
T. aeria (Pa-3) §	*T. aeria* (Pa-3) §	*T. aeria* (Pa-3) §	*T. aeria* (Pa-3) §
T. isobilateralis‡	*T. isobilateralis*‡	*T. isobilateralis*‡	*T. isobilateralis*‡
T. dissociata‡	*T. dissociata*‡	*T. dissociata*‡	*T. dissociata*‡
T. illinoisensis‡	*T. illinoisensis*‡	*T. illinoisensis*‡	*T. illinoisensis*‡
T. aggregata‡	*T. aggregata*‡	*T. aggregata*‡	*T. aggregata*†
*T. aplanosporum**	*T. aplanosporum**	*T. aplanosporum*†	*T. aplanosporum**
*T. pampae**	*T. pampae**	*T. pampae*†	*T. pampae**
		T. intermedium†	
T. tetrasporum† to*	*T. tetrasporum*¶	*T. tetrasporum**	*T. tetrasporum*§
			T. intermedium§
			T. excentrica† to ‡
T. excentrica†	*T. excentrica*‡	*T. excentrica*‡	
T. texensis†	*T. texensis*‡	*T. texensis*‡	*T. texensis*‡
T. pulchra‡	*T. pulchra*‡		*T. pulchra*‡
T. intermedium‡	*T. intermedium*‡		
		T. pulchra¶	

* = distinct (very little relation)
† = some degree of relation
‡ = very close relation
§ = identical
¶ no data

intermedium and ***T. tetrasporum*** than to ***T. texensis*** or ***T. pulchra.*** Thus, serological and ultrastructural data may be of value in showing the way in which the 4 species of this group may be related, possibly through a serological similarity to ***T. excentrica.***

VI. Summary

This paper summarizes a comparative investigation of taxa in the chlorophycean genera *Tetracystis* and *Chlorococcum* using morphological (light and electron-microscopic), physiological, and serological techniques. The following are salient results and conclusions:

1. A new chlorosphaeralean genus *Tetracystic*, typified by **T. aeria** (C–6), has been erected. Eight additional species listed below have also been described:

T. aggregata sp. nov.
T. dissociata sp. nov.
T. excentrica sp. nov.
T. illinoisensis sp. nov.
T. isobilateralis sp. nov.
T. pampae sp. nov.
T. pulchra sp. nov.
T. texensis sp. nov.

Herbarium specimens of these taxa have been deposited in the Chicago Natural History Museum and cultures of the living organisms have been sent to the Culture Collection of Algae, Indiana University, Bloomington, Indiana.

2. Three taxa, formerly treated by other investigators as species of the genus *Chlorococcum*, have been transferred to the genus *Tetracystis* on the basis of new data obtained in this investigation. These are:

Tetracystis aplanosporum (Arce and Bold) Brown and Bold comb. nov.
T. intermedium (Deason and Bold) Brown and Bold comb. nov.
T. tetrasporum (Arce and Bold) Brown and Bold comb. nov.

3. A key to these 12 species of *Tetracystis* has been prepared.

4. Electron-microscopic studies have yielded comparative data which are of taxonomic value in distinguishing species of *Tetracystis*. These data include differences in organization of the chloroplast, pyrenoid, mitochondria, Golgi apparatus, and cell wall.

5. The electron-microscopic studies have provided insight into the course and mechanism of pyrenoid division and the behavior of the pyrenoid during zoosporogenesis and vegetative cell division. Furthermore, electron microscopy has provided a firm basis for distinguishing the phenomena related to vegetative cell division and zoosporogenesis, as these occur in the Chlorosphaerales and Chlorococcales.

6. Serological investigations have supported taxonomic groupings originally based on morphological and physiological criteria alone. In addition, these data have revealed serological intergeneric specificity as well as common antigenicity between *Chlorococcum* and *Tetracystis*.

7. On the basis of the investigations herein reported, which employed diverse techniques, it is concluded that the orders Chlorosphaerales, as exemplified by *Tetracystis*, and Chlorococcales, as represented by *Chlorococcum*, are probably validly segregated.

8. A summary of species groupings based on morphological, ultrastructural, and immunochemical data is presented in Table 27.

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Fig. 1–8. *Tetracystis excentrica*.—Fig. 1. Mature vegetative cells; note excentric pyrenoid.—Fig. 2. Mature vegetative cells, some of which are undergoing vegetative cell division.—Fig. 3. Mature tetrad of daughter vegetative cells. Note loose association of parent wall with the daughter cells.—Fig. 4–8[a]. Sexual reproduction. Photographs made at 5-sec intervals. Note spherical cells (young zygotes). All ×1300.

[a] **See also Fig. 9–11.**

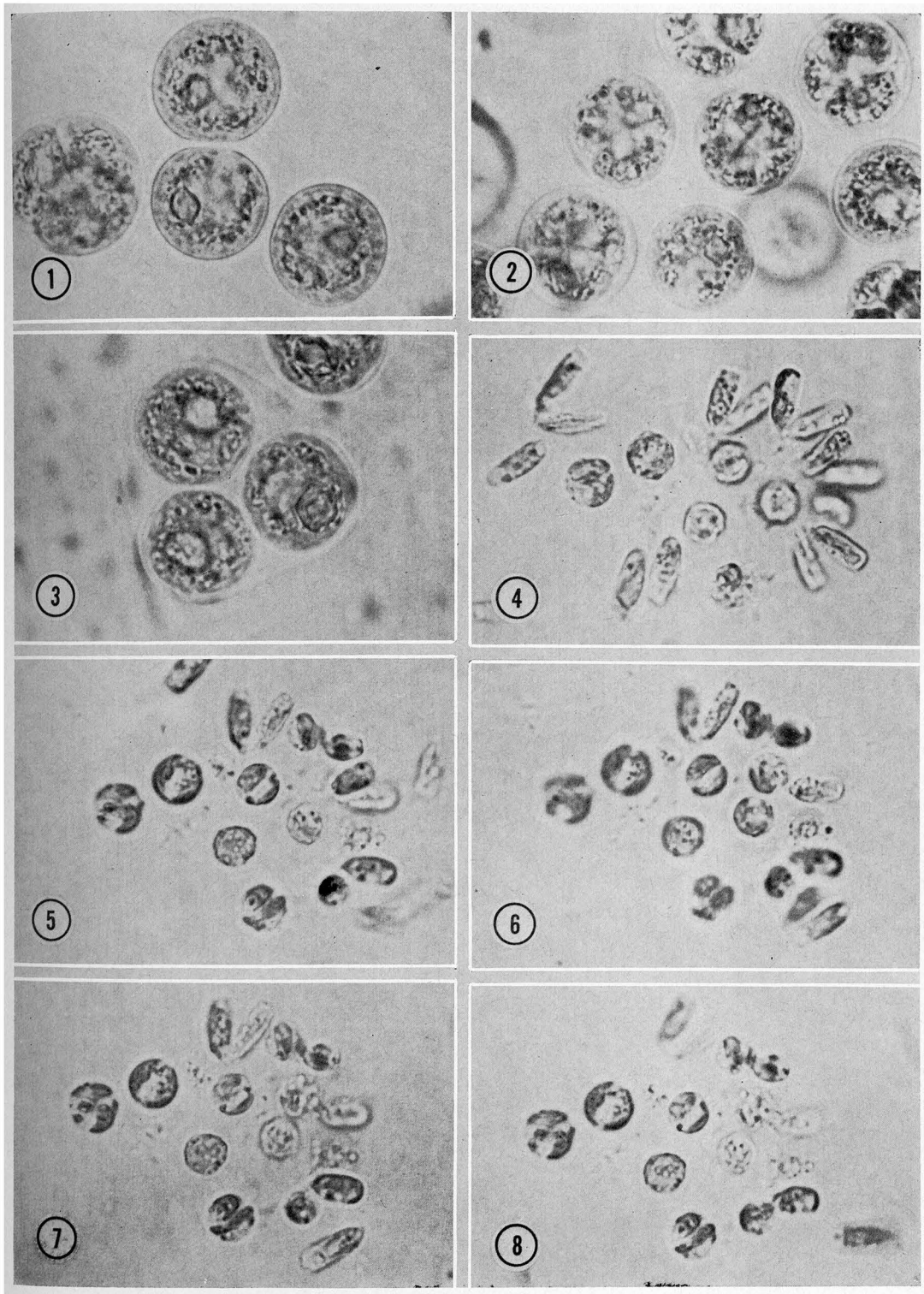
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Fig. 9–12. *Tetracystis excentrica.*—Fig. 9–11. Sexual reproduction, continued.—Fig. 12. Giant cells, possibly zygotes. Note unequal cleavage. Also note young vegetative cells.

Fig. 13–16.*Tetracystis aeria* (C–6).—Fig. 13. Young vegetative cells.—Fig. 14. Mature vegetative cell; note nucleus.—Fig. 15. Mature vegetative cells and tetrahedral tetrads.—Fig. 16. Mature vegetative cell (at right) during early zoosporogenesis.

All photos, with the exception of Fig. 14, ×1300; Fig. 14, ×2000.

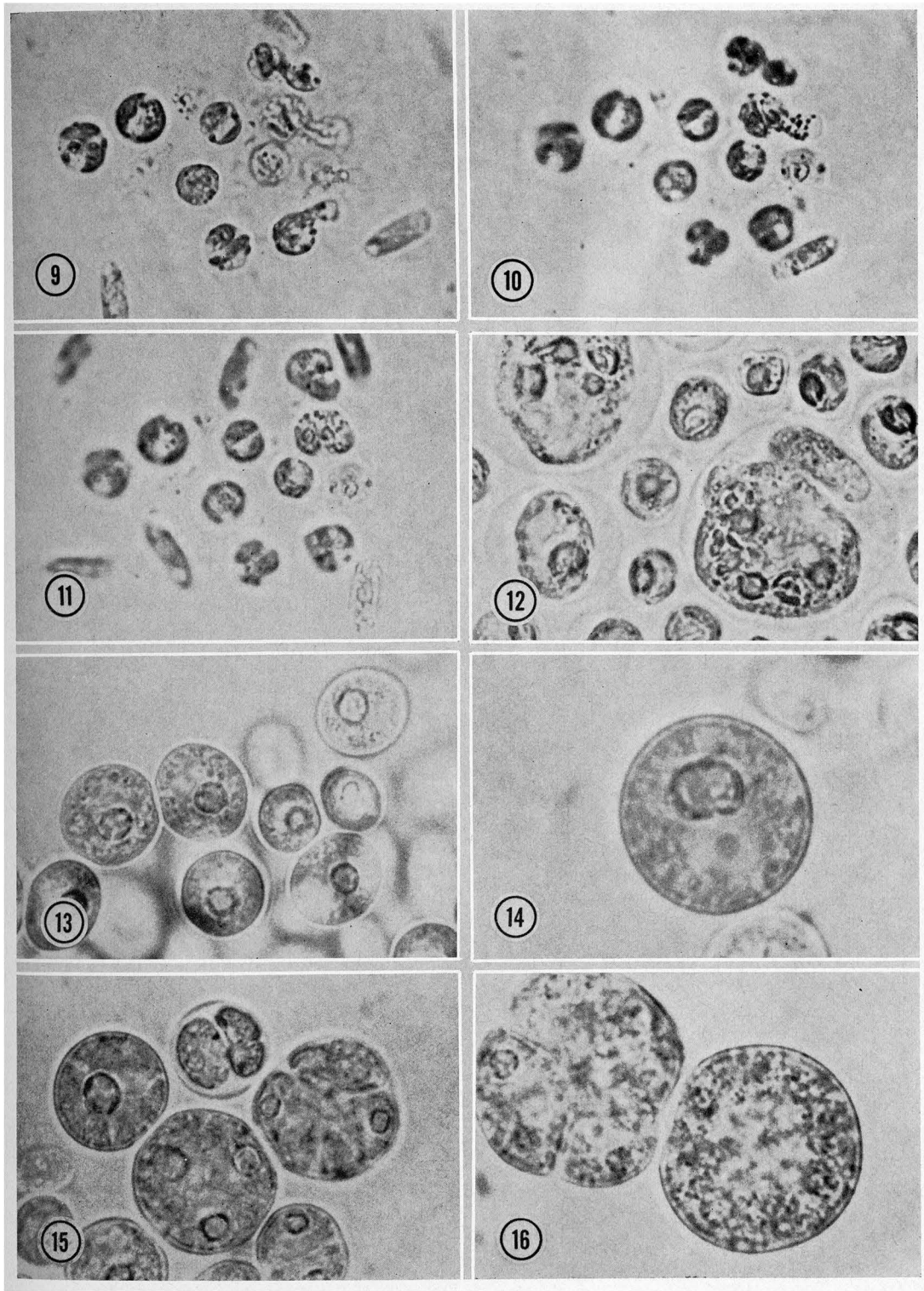
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Fig. 17–18. *Tetracystis aeria* (continued).—Fig. 17. Later zoosporogenesis.—Fig. 18. Zoosporangium, prior to release of zoosporees.

Fig. 19–24. *Tetracystis texensis*.—Fig. 19. Young and mature vegetative cells.—Fig. 20. Early octad (left) and tetrad (right) vegetative cells.—Fig. 21. Mature vegetative cell (bottom) and early tetrad of cells.—Fig. 22. Tetrad complex.—Fig. 23. Zoosporangium with 8 zoospores.—Fig. 24. Aplanosporangium (left) and tetrad (right). All $\times$1300.

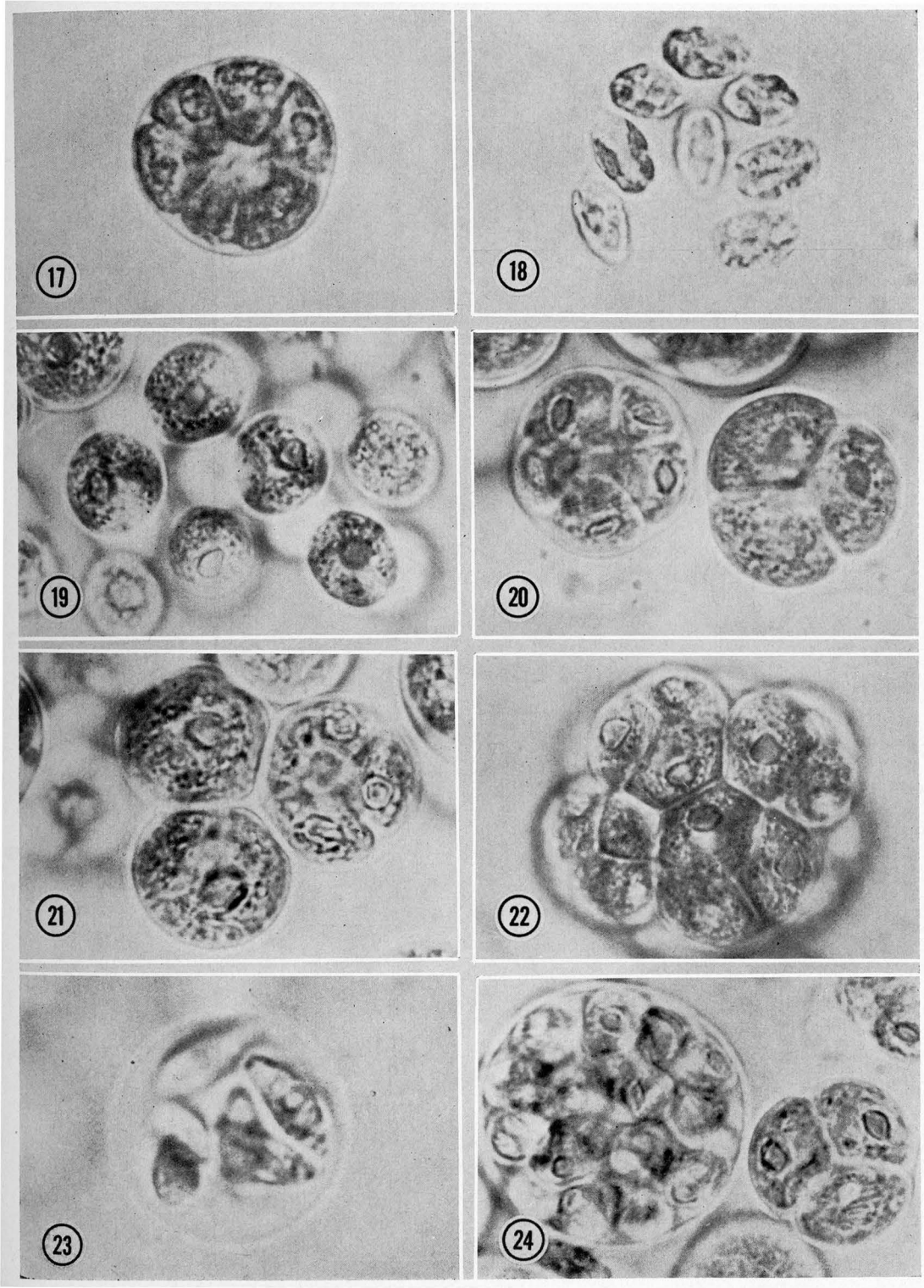
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Fig. 25–32. *Tetracystis dissociata*.—Fig. 25. Young and mature vegetative cells.—Fig. 26. Mature vegetative cells. Note irregularly shaped central pyrenoid and radial fissures in chloroplast.—Fig. 27. Early diad stage.—Fig. 28. Diad undergoing division to form isobilateral tetrad. Fig. 29. Tetrahedral tetrad complexes.—Fig. 30. Zoosporangium with 16 zoospores.—Fig. 31. Zoospore immediately upon quiescence.—Fig. 32. Aplanosporangium about to release aplanospores. All ×1300.

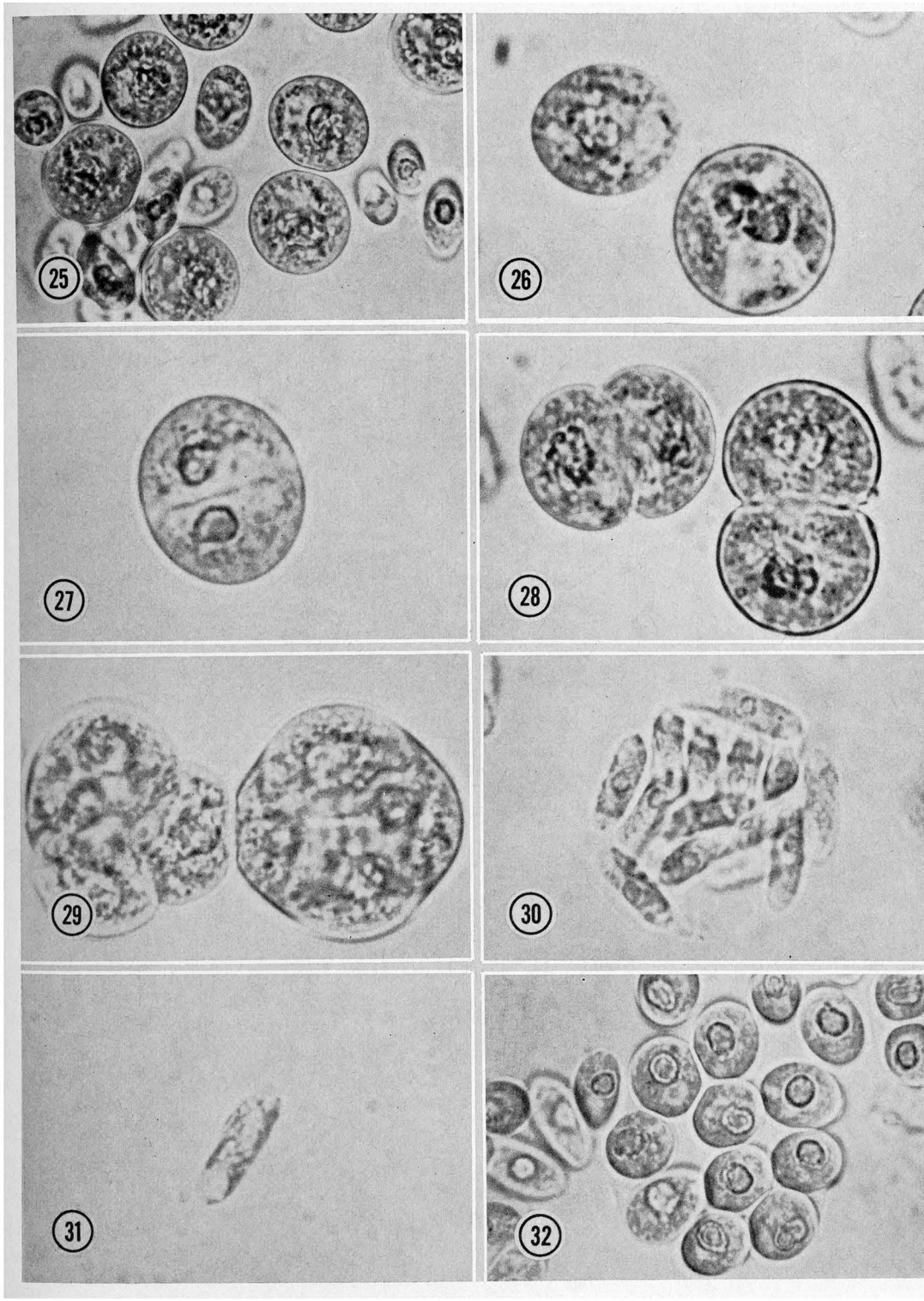
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Fig. 33–40. ***Tetracystis aggregata.*****—Fig. 33. Young vegetative cells.—Fig. 34. Mature vegetative cell and young tetrads.—Fig. 35. Complexes of isobilateral and tetrahedral tetrads.—Fig. 36. Early zoosporogenesis.—Fig. 37. Daughter cells of the tetrad undergoing zoospore formation.—Fig. 38. Zoospore at quiescence. Note flagella which are longer than cell body length, and posterior nucleus.—Fig. 39. Aplanosporangium.—Fig. 40. Echinate zygotes among young and mature vegetative cells. All × 1300.**

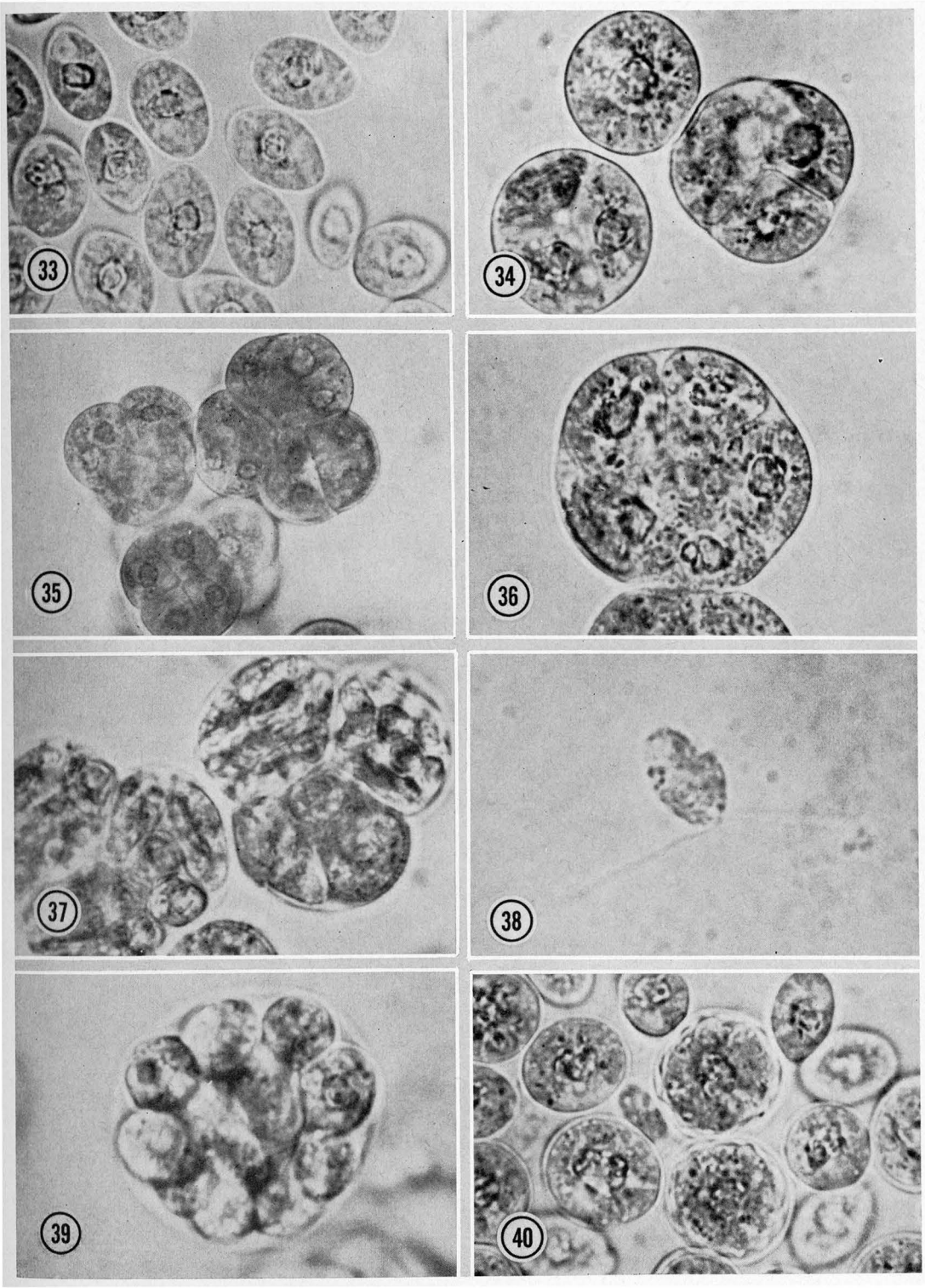
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Fig. 41–48. *Tetracystis isobilateralis*.—Fig. 41. Young vegetative cells.—Fig. 42. Mature vegetative cells; note massive chloroplast.—Fig. 43. Complex of 2 isobilateral tetrads.—Fig. 44. Tetrahedral tetrad undergoing further vegetative cell division.—Fig. 45. A tetrad of zoosporangia.—Fig. 46. Zoospore at quiescence. Note flagellum (longer than cell body in length), parietal chloroplast, and posterior nucleus.—Fig. 47. Echinate zygote.—Fig. 48. Zygote germination. All ×1300.

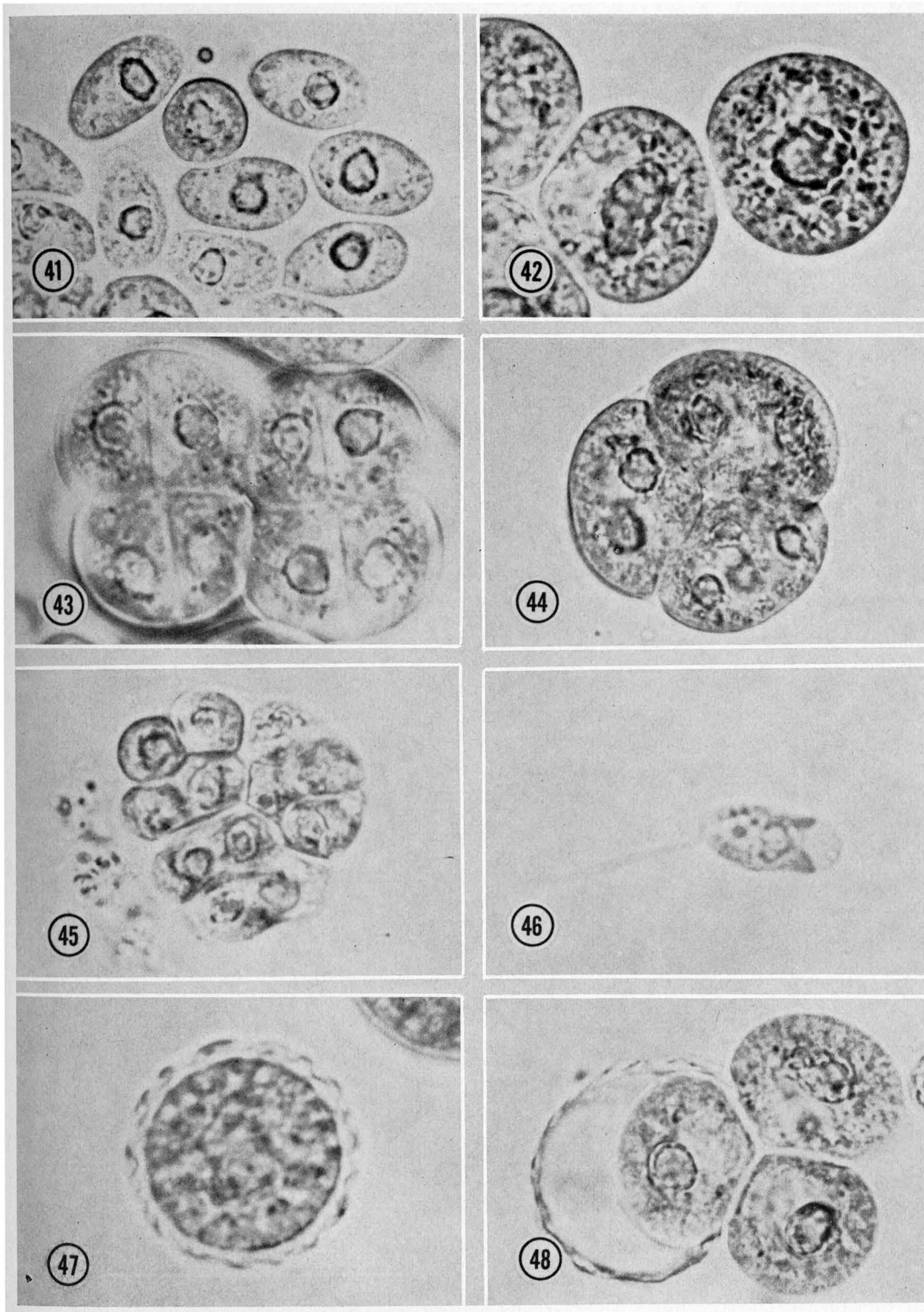
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Fig. 49–52. ***Tetracystis pulchra.*****—Fig. 49. Young and mature vegetative cells.—Fig. 50. Tetrads of vegetative cells and zoosporangium.—Fig. 51. Mature vegetative cells. Note polar wall thickenings.—Fig. 52. Mature vegetative cell (upper right) with contractile vacuole.**

Fig. 53–56. ***Tetracystis illinoisensis.*****—Fig. 53. Young and mature vegetative cells.—Fig. 54. Early diad formation.—Fig. 55. Mature diads.—Fig. 56. Zoosporangium. All ×1300.**

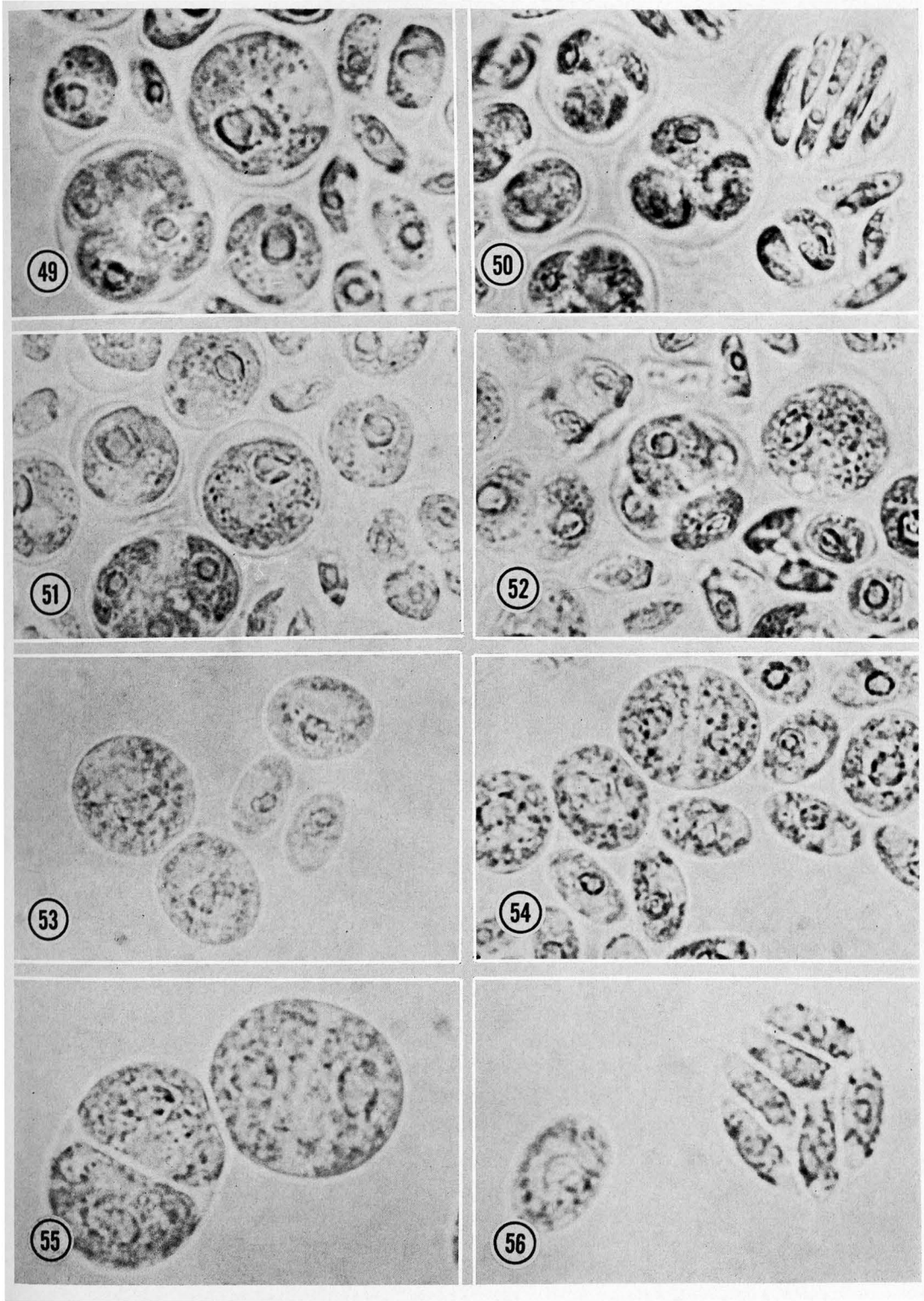
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Fig. 57–64. *Tetracystis pampae*.—Fig. 57. Mature, ellipsoidal, vegetative cells.—Fig. 58. Tetrads of vegetative cells.—Fig. 59. Tetrads of cells in early zoosporogenesis.—Fig. 60. Tetrahedral tetrads viewed from 2 different positions.—Fig. 61. Early zoosporogenesis of mature vegetative cells.—Fig. 62. Aplanosporangium containing zoosporangia with 2 zoospores each.—Fig. 63. Young vegetative cells and single zoosporangia with 2 zoospores each.—Fig. 64. Aplanosporangia with 2 aplanospores in each. All ×1300.

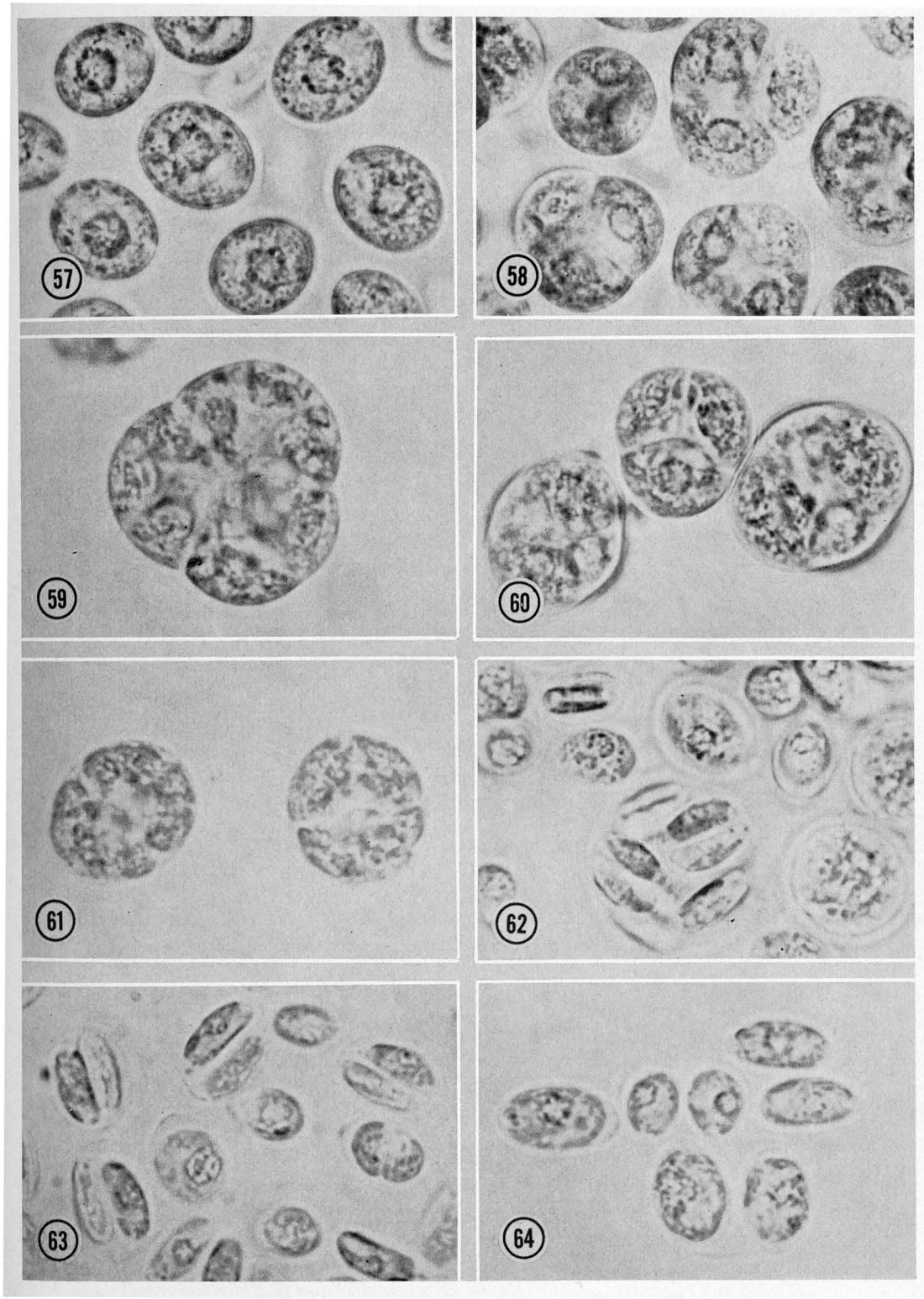
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Fig. 65–72. *Tetracystis aplanosporum*.—Fig. 65. Young vegetative cells.—Fig. 66. Mature vegetative cells. Note contractile vacuoles (arrow).—Fig. 67 Early tetrad formation.—Fig. 68. Tetrads of vegetative cells.—Fig. 69. Very early zoosporogenesis in cell at left in which the pyrenoid has "disappeared"; chloroplast and cytoplasmic furrowing are in progress in the cell at the right.—Fig. 70. Later zoosporogenesis.—Fig. 71. Zoosporangium.—Fig. 72. Aplanosporangium. All ×1300.

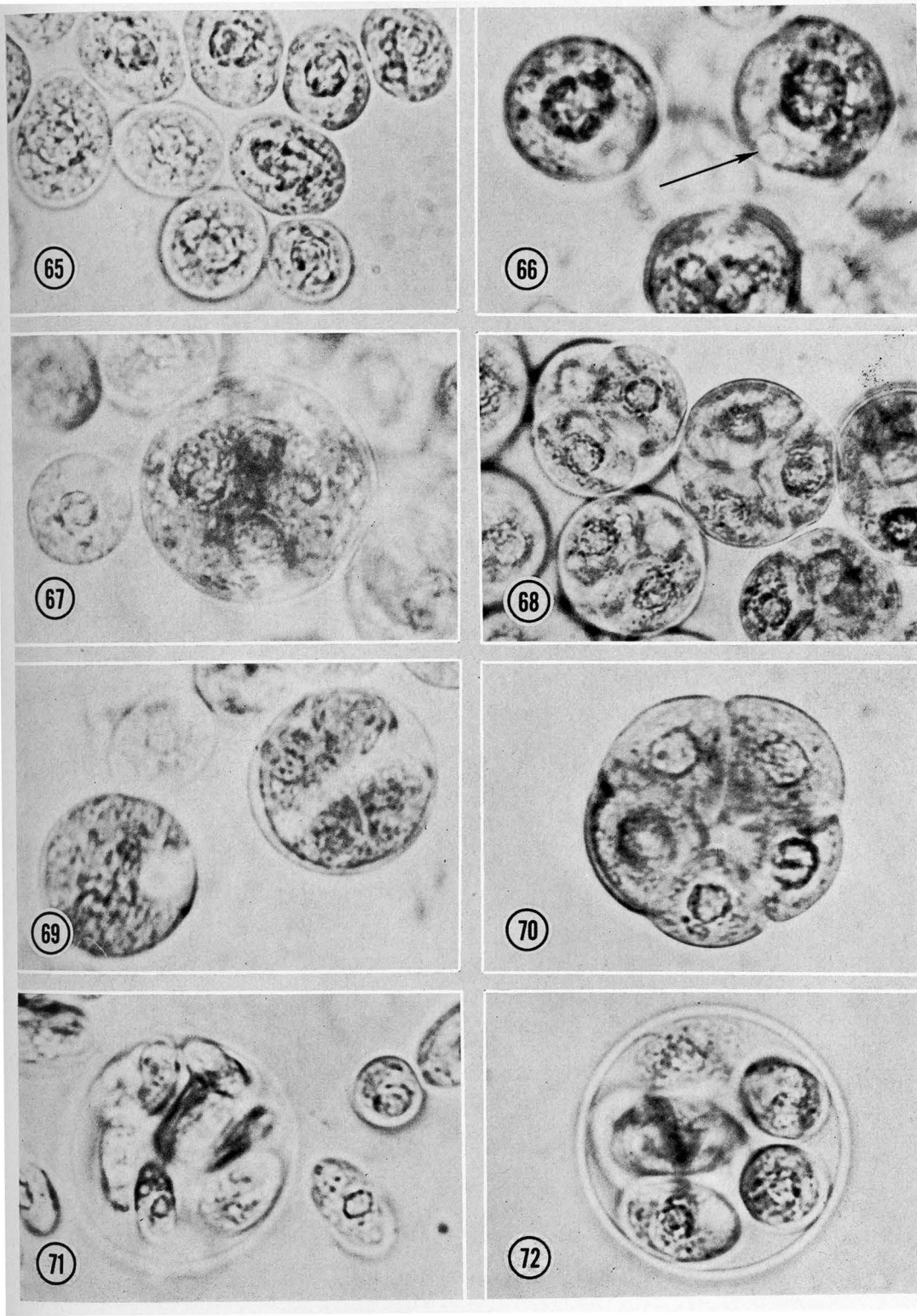
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Fig. 73–78. *Tetracystis intermedium*.—Fig. 73. Young and mature vegetative cells.—Fig. 74. Mature vegetative cell in process of forming a diad at left of photo.—Fig. 75. Tetrads of vegetative cells.—Fig. 76. Complex of tetrads.—Fig. 77. Zoosporangium.—Fig. 78. Young and mature vegetative cells; aplanosporangium at right of photo.

Fig. 79–80. *Tetracystis tetrasporum*.—Fig. 79. Young and mature vegetative cells. Note contractile vacuole in mature vegetative cell.—Fig. 80. Tetrads of vegetative cells. All ×1300.

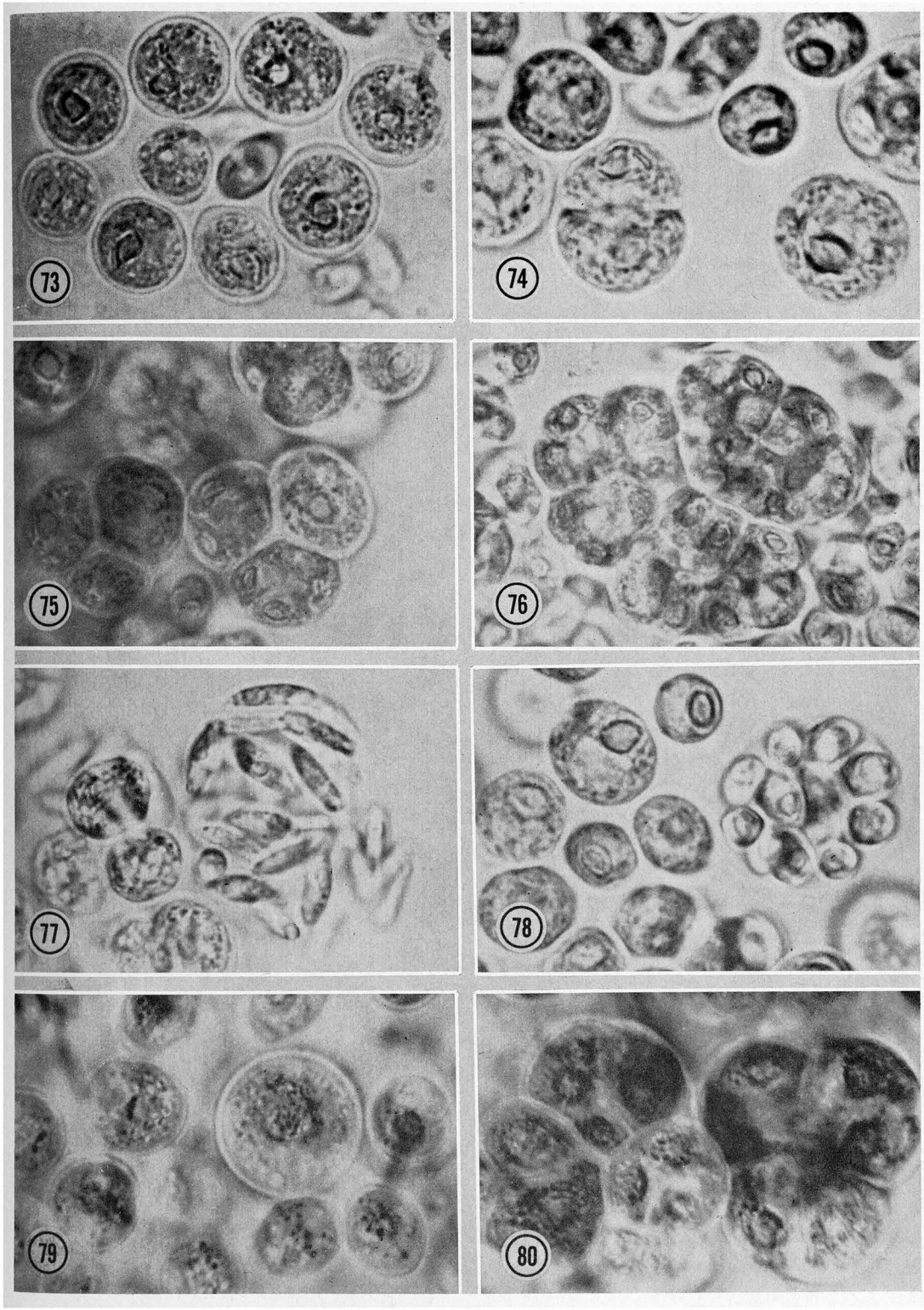
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Fig. 81–94. ***Tetracystis*** **species at 1 and 2 weeks on BBM agar.—Fig. 81.** ***T. isobilateralis,*** **2 weeks.—Fig. 82.** ***T. isobilateralis,*** **1 week.—Fig. 83.** ***T. aggregata,*** **2 weeks.—Fig. 84.** ***T. aggregata,*** **1 week.—Fig. 85.** ***T. illinoisensis,*** **2 weeks.—Fig. 86.** ***T. illinoisensis,*** **1 week.—Fig. 87.** ***T. dissociata,*** **2 weeks.—Fig. 88.** ***T. dissociata,*** **1 week—Fig. 89.** ***T. aeria*** **(C–6), 2 week.—Fig. 90.** ***T. aeria*** **(C–6), 1 week.—Fig. 91.** ***T. aeria*** **(Pa–3), 2 weeks.—Fig. 92.** ***T. aeria*** **(Pa–3), 1 week.—Fig. 93.** ***T. tetrasporum,*** **2 weeks.—Fig. 94.** ***T. tetrasporum,*** **1 week. 2 weeks, ×45; 1 week, ×65.**

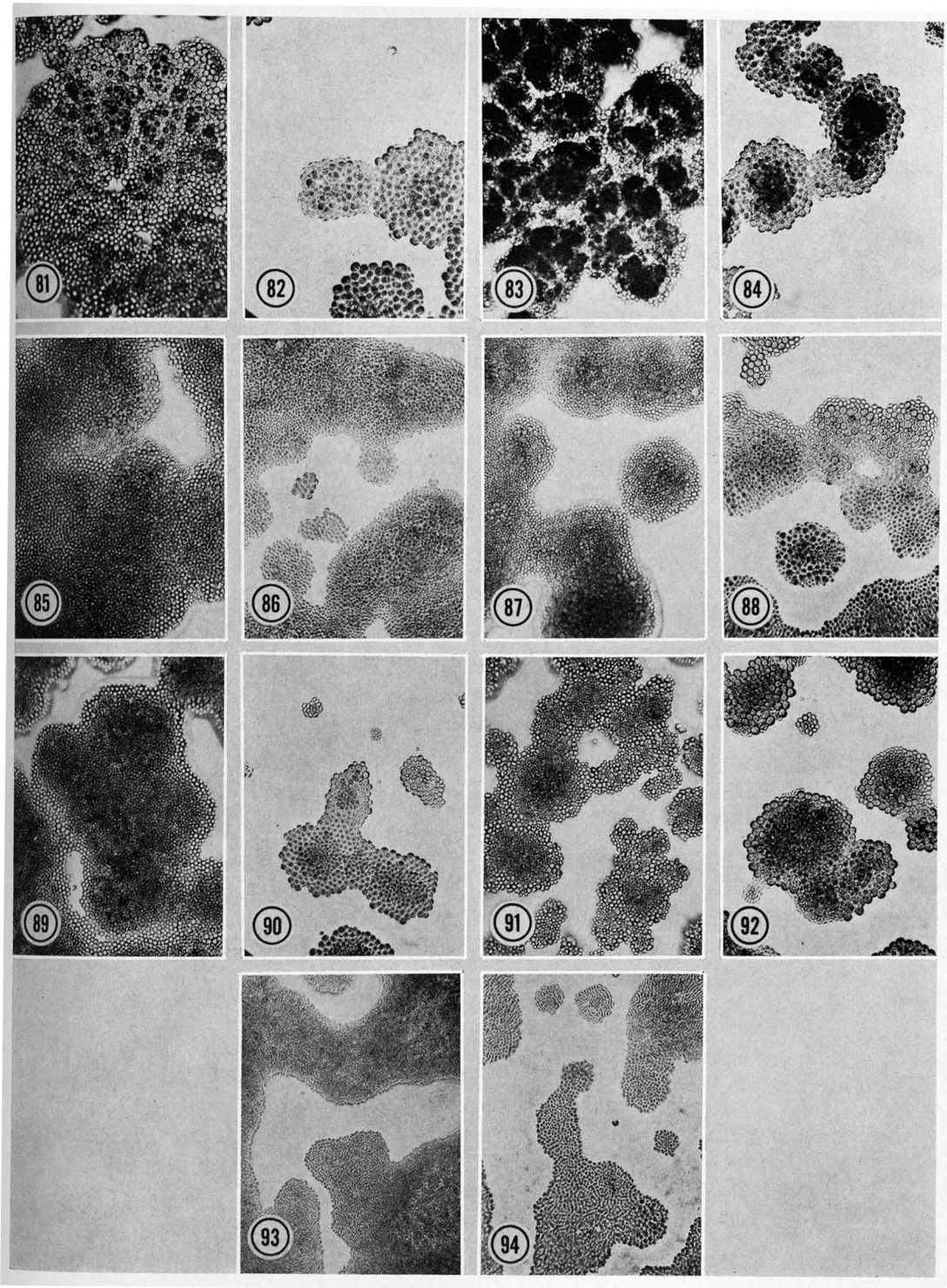
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Fig. 95–106. ***Tetracystis*** **species at 1 and 2 weeks on BBM agar (continued).—Fig. 95.** ***T. texensis*****, 2 weeks.—Fig. 96.** ***T. texensis*****, 1 week.—Fig. 97.** ***T. pulchra*****, 2 weeks.—Fig. 98.** ***T. pulchra*****, 1 week.—Fig. 99.** ***T intermedium*****, 2 weeks.—Fig. 100.** ***T. intermedium*****, 1 week.—Fig. 101.** ***T. excentrica*****, 2 weeks.—Fig. 102.** ***T. excentrica*****, 1 week.—Fig. 103.** ***T. aplanosporum*****, 2 weeks.—Fig. 104.** ***T. aplanosporum*****, 1 week.—Fig. 105.** ***T. pampae*****, 2 weeks.—Fig. 106.** ***T. pampae*****, 1 week. 2 weeks, ×45; 1 week, ×65.**

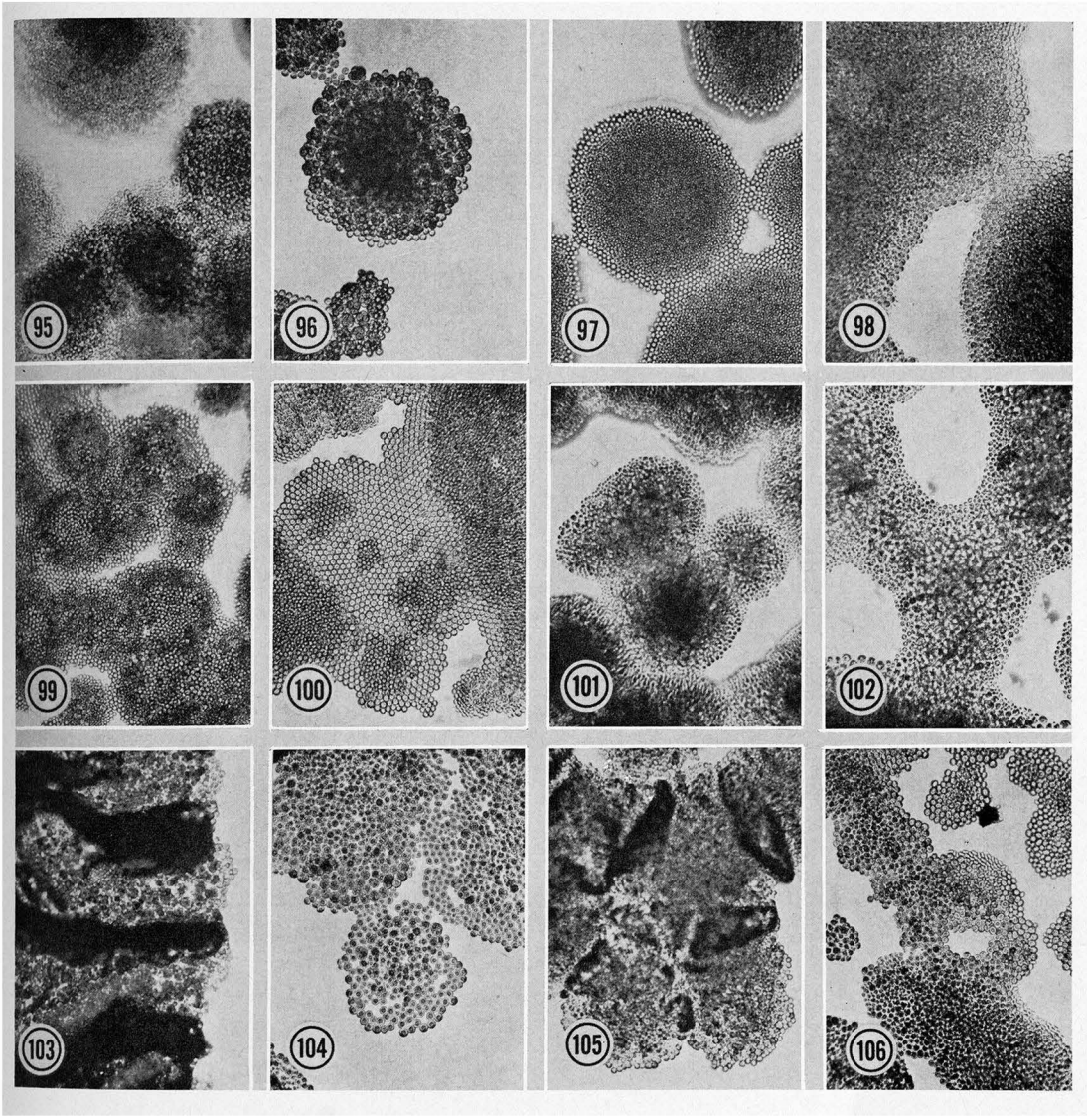
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Fig. 107. Growth of *Tetracystis* species (at 2 weeks) on BBM agar with crystal violet at a concentration of 1/100,000. No. 1, *T. texensis;* No. 2, *T. aggregata;* No. 3, *T. aeria* (Pa–3); No. 4, *T. isobilateralis;* No. 5, *T. excentrica;* No. 6, *T. aeria* (C–6); No. 7, *T. dissociata;* No. 8, *T. pampae;* No. 9, unidentified species of *Trebouxia;* No. 10, *T. tetrasporum;* No. 11, *T. aplanosporum;* No .12, *T. intermedium.*

Fig. 108. Amylasic activity of *Tetracystis* species on BBM supplemented with 0.01 % soluble starch and grown for 2 weeks under standard conditions. No. 1, *T. isobilateralis;* No. 2, *T. pampae;* No. 3, *T. aggregata;* No. 4, *T. tetrasporum;* No. 5, *T. excentrica;* No. 6, *T. aeria* (C–6); No. 7, *T. aeria* (Pa–3); No. 8, *T. texensis;* No. 9, *T. dissociata;* No. 10, unknown species of *Trebouxia;* No. 11, *T. aplanosporum;* No. 12, *T. intermedium.*

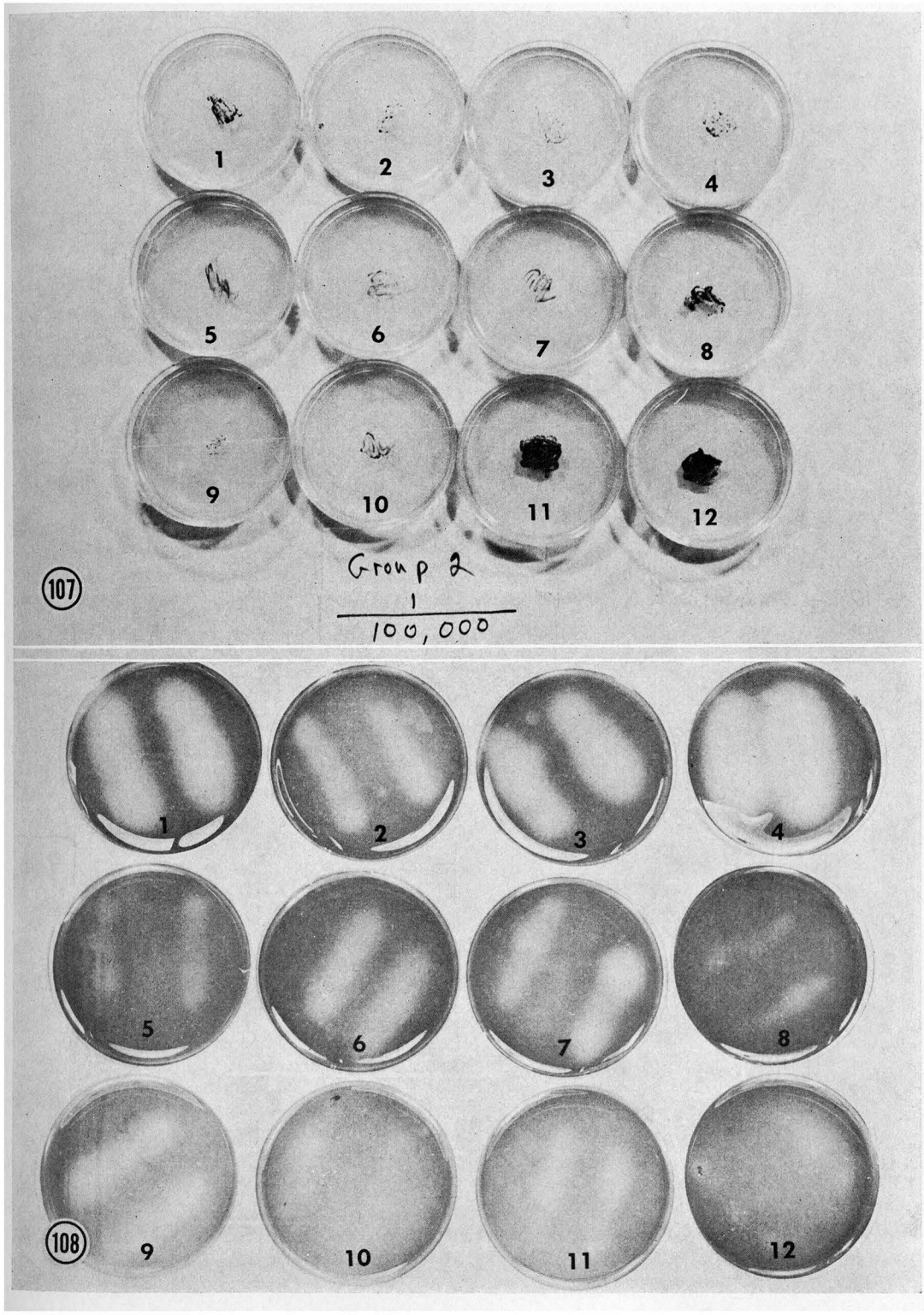
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Fig. 109–114[a]. Various permanganate fixation images of the pyrenoid and surrounding area of *Tetracystis isobilateralis*. All material fixed for 1 hr, 10 min at 5° C.—Fig. 109. 2% $LiMnO_4$, ×28,000.—Fig. 110. 2% $KMnO_4$, ×24,200.—Fig. 111. 2% $NaMnO_4$, ×24,200.—Fig. 112 2% $Ca(MnO_4)_2$, × 17,500. Arrow 1; note the swollen pyrenoid lamellae, peculiar to the divalent cation permanganate fixatives used.—Fig. 113. 2% $Mg(MnO_4)_2$, ×11,400.—Fig. 114. 2% $Sr(MnO_4)_2$, ×16,400. Arrow 2 indicates the granular nature of the pyrenoid matrix, obtained only with 2% $Sr(MnO_4)_2$.

[a] **Unless otherwise indicated, all material for electron-microscopy was fixed at 2% $LiMnO_4$ (aqueous) at 5° C for 2 hr.**

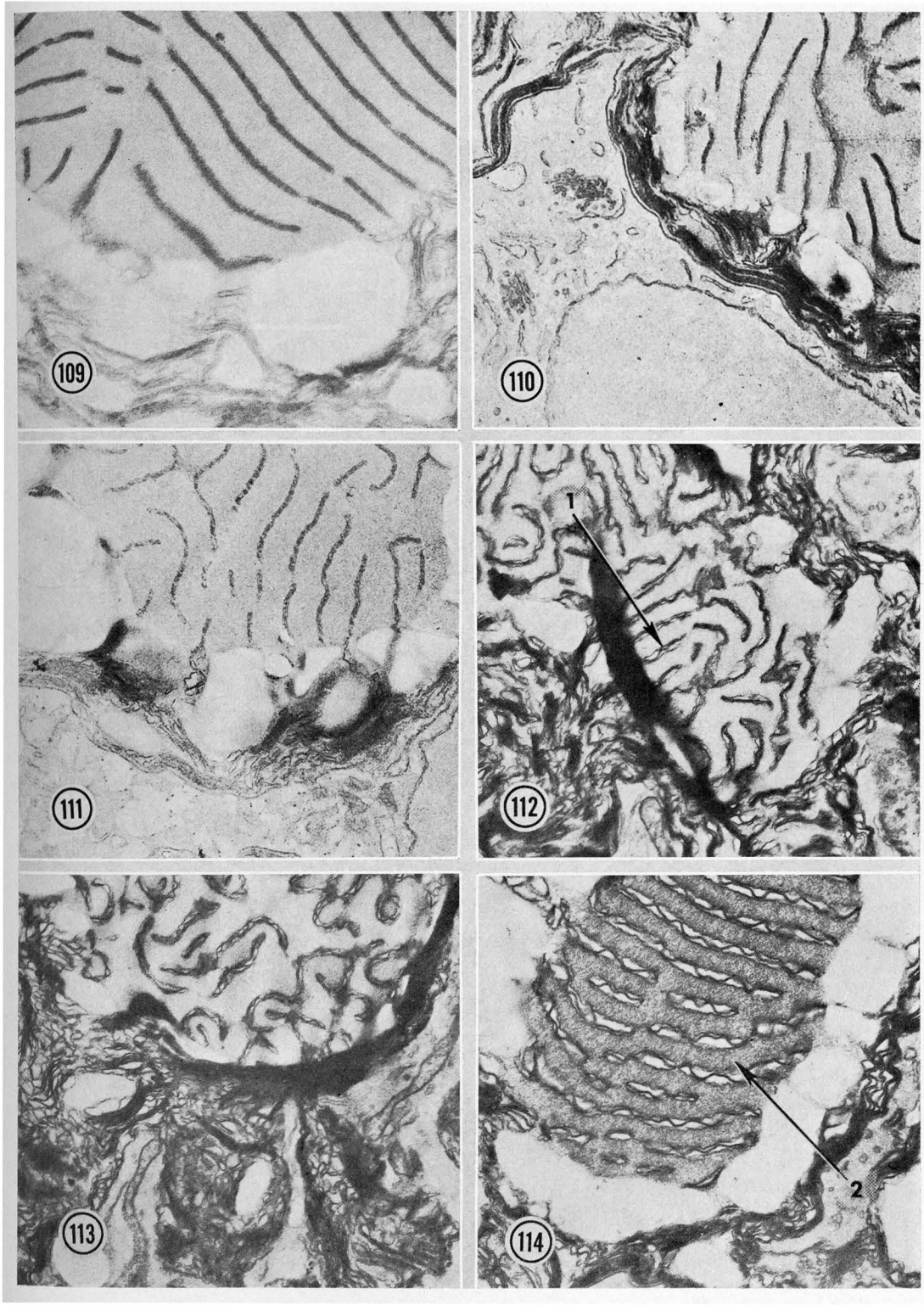
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Fig. 115–116. Glutaraldehyde fixation study of *Tetracystis isobilateralis*.—Fig. 115. Material fixed in glutaraldehyde for 16 days, then treated with 1% OsO_4. Arrows indicate the nucleolus and "osmiophilic granules" of the chloroplast, ×26,000. —Fig. 116. Material from same glutaraldehyde treatment as above but subsequently treated with 2% $LiMnO_4$. Arrows indicate nucleolus and "osmiophilic granules" of the chloroplast not preserved with 2% $LiMnO_4$ alone, ×16,200.

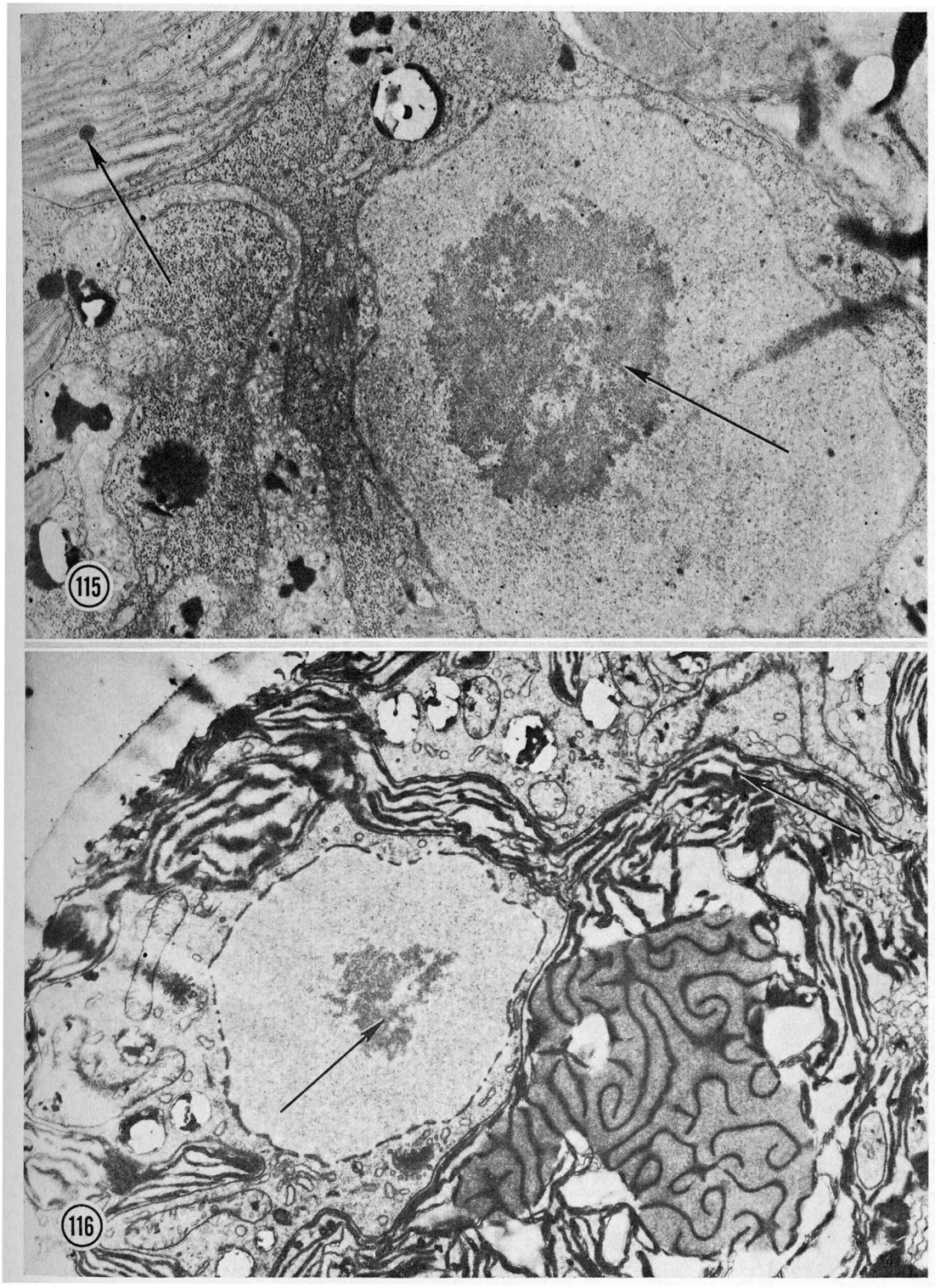
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Fig. 117–120. Glutaraldehyde fixation study of *Tetracystis isobilateralis (continued).*—Fig. 117. Post-treatment with 1 % OsO_4. Arrow 1 shows the lack of ribosomes on the side of the endoplasmic reticulum facing the Golgi apparatus. Arrow 2 indicates electron-dense material preserved in the vacuole. Arrow 3 shows ribosomes of the ground cytoplasm. Arrow 4 indicates Golgi cisternal elements, ×24,600. Fig. 118. Post-treatment with 1 % OsO_4 showing general fixation image of the chloroplast. Arrow 5 indicates pyrenoid lamellae, ×24.600.—Fig. 119. Post-treatment with 2 % $LiMnO_4$. Arrow 6 shows ribosome-like particles of the ground cytoplasm. Arrow 7 indicates electron-dense staining material in the vacuole, not so stained in material fixed in $LiMnO_4$ alone, ×28,900.—Fig. 120. Post-treatment with 2 % $LiMnO_4$ showing general fixation image of the chloroplast. Arrow 8 indicates pyrenoid lamellae. Compare with the post-treatment with OsO_4 (Fig. 118) and note the greater contrast between matrix and lamellae in Fig. 120, ×23,800.

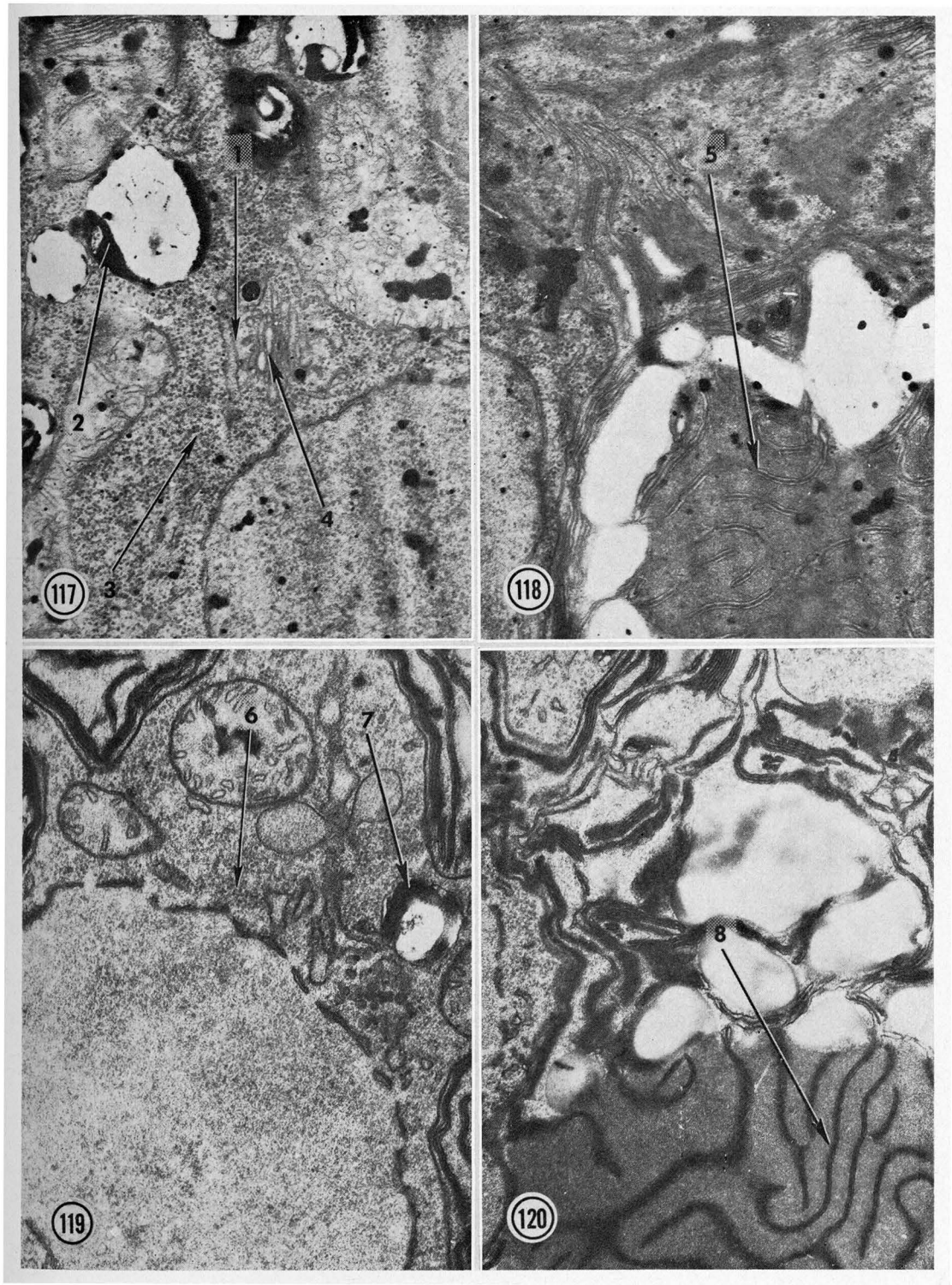
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Fig. 121–123. ***Tetracystis aeria*** **(C–6).—Fig. 121. Mature vegetative cell. Note invagination of chloroplast (arrow 3), thick outer wall (arrow 1), reticulations of the chloroplast lamellae (arrow 2), and cylindrical mitochondrion (arrow 4), ×10,300.—Fig. 122. Tetrahedrad tetrad. Note lack of distinct Golgi amplexi (arrow 5), ×5,700.—Fig. 123. Portion of a tetrad. Note variable stacking (from 2 to 6 to 8) of lamellae in chloroplast (arrow 6) and undifferentiated elements of the Golgi cisternae (arrow 7), ×15,400.**

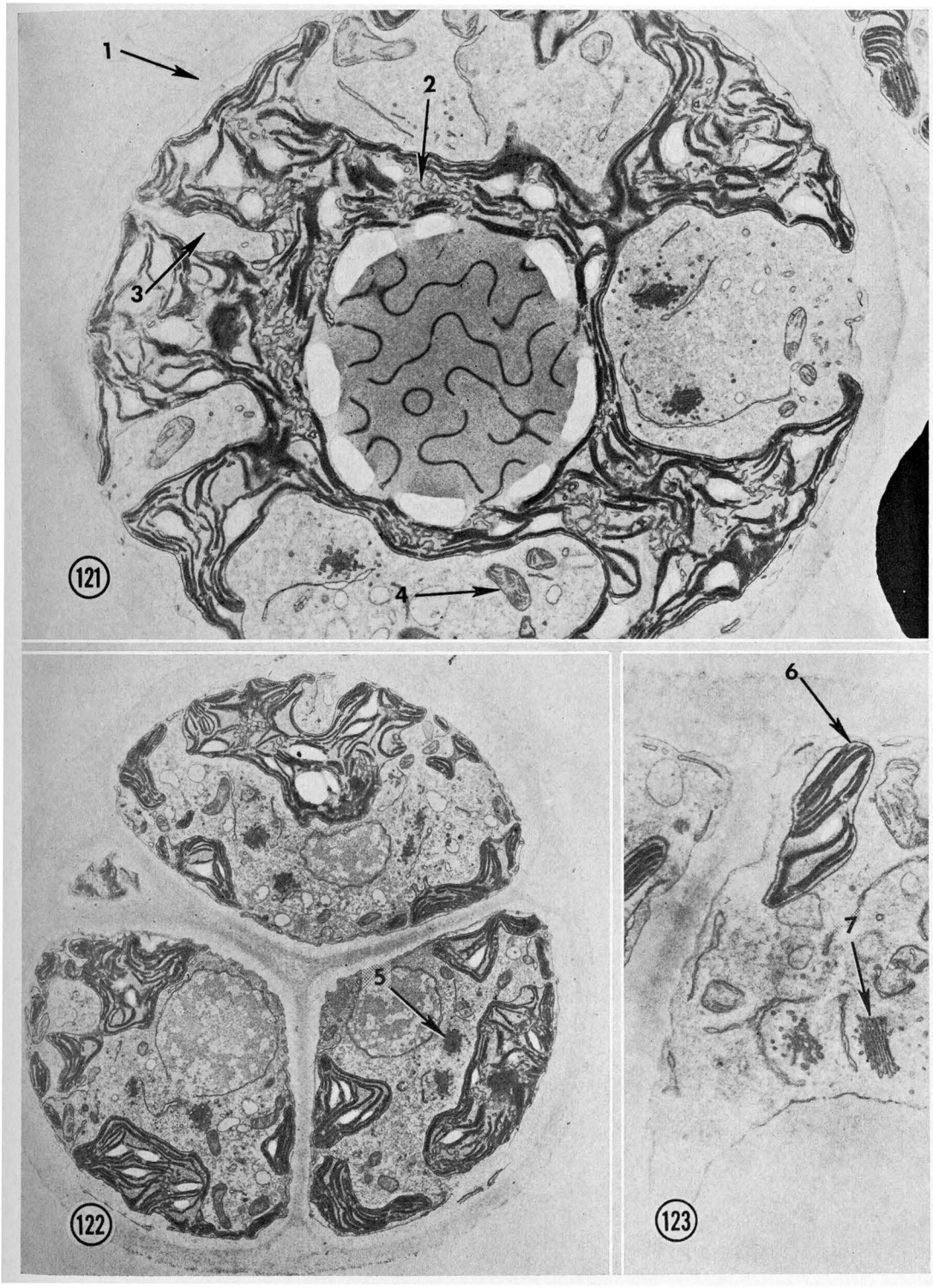
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Fig. 124–126. ***Tetracystis aeria*** **(Pa–3).—Fig. 124. Vegetative cell. Arrow 1 indicates thick, electron-dense wall layer. Arrow 2 shows non-inflated Golgi apparatus which lacks distinct amplexus. Arrow 3 refers to typical chloroplast invaginations, ×10,400.—Fig. 125. Portion of chloroplast showing lamellar stacking (arrow 4), ×21,200.—Fig. 126. Early-formed tetrad of daughter vegetative cells. Compare thickness of electron-dense layer of daughter cells with that of parent. Note cylindrical mitochondrion (arrow 5) and reticulations of chloroplast (arrow 6), ×5600.**

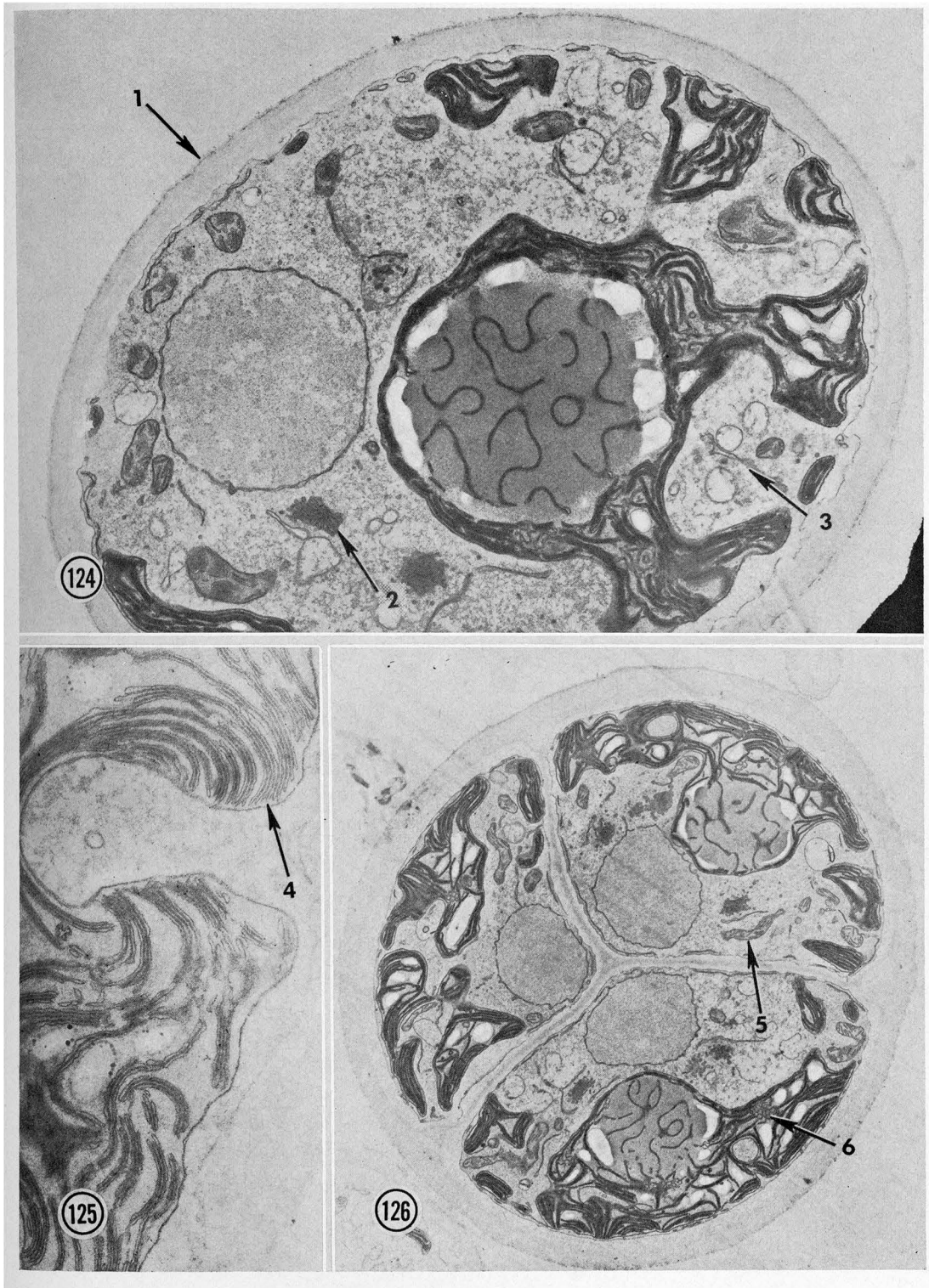
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Fig. 127–129. ***Tetracystis isobilateralis.***—**Fig. 127. Mature isobilateral tetrad of daughter vegetative cells. Note the wall relation of the daughter cells (arrow 1) which indicates that intervening diad formation has occurred, ×7000.—Fig. 128. Portion of vegetative cell showing chloroplast lamellar stacking (arrow 2) and thick, inner, granular cell wall layer, ×13,000.—Fig. 129. Unknown organelle (arrow 4) often present in this species; chloroplast showing variable stacking of lamellar components (arrow 3), ×15,000.**

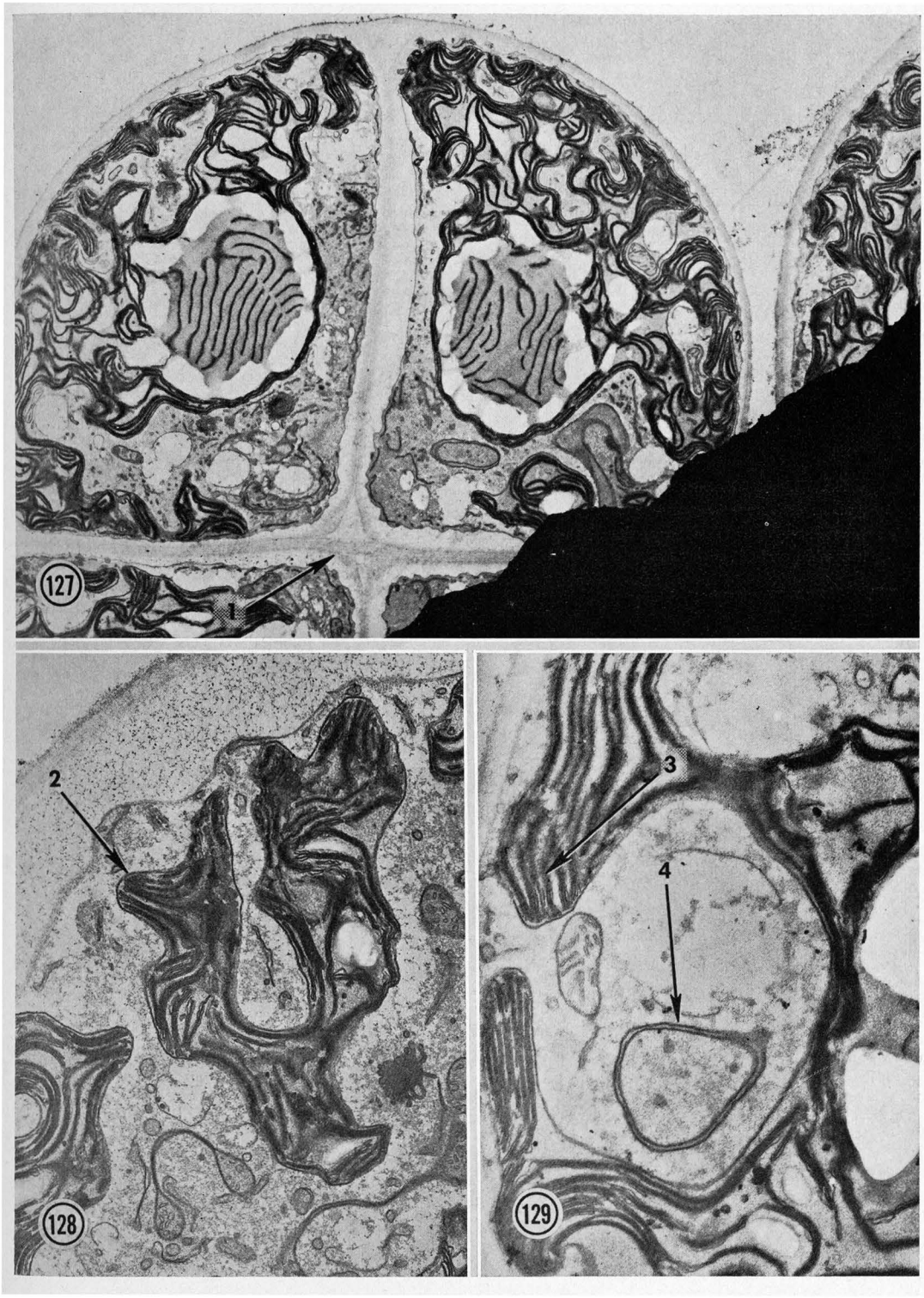
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Fig. 130–131. ***Tetracystis isobilateralis*** **(continued).—Fig. 130. Mature vegetative cell with thick, inner wall layer (arrow 1), massive chloroplast with centrifugal (arrow 2) and centripetal (arrow 3) fissuring, ×8,000.—Fig. 131. Diad of daughter vegetative cells with giant, branched, compressed mitochondrion (arrow 5) and Golgi apparatus without distinct amplexi, cisternal elements of which are uninflated (arrow 4), ×7,800.**

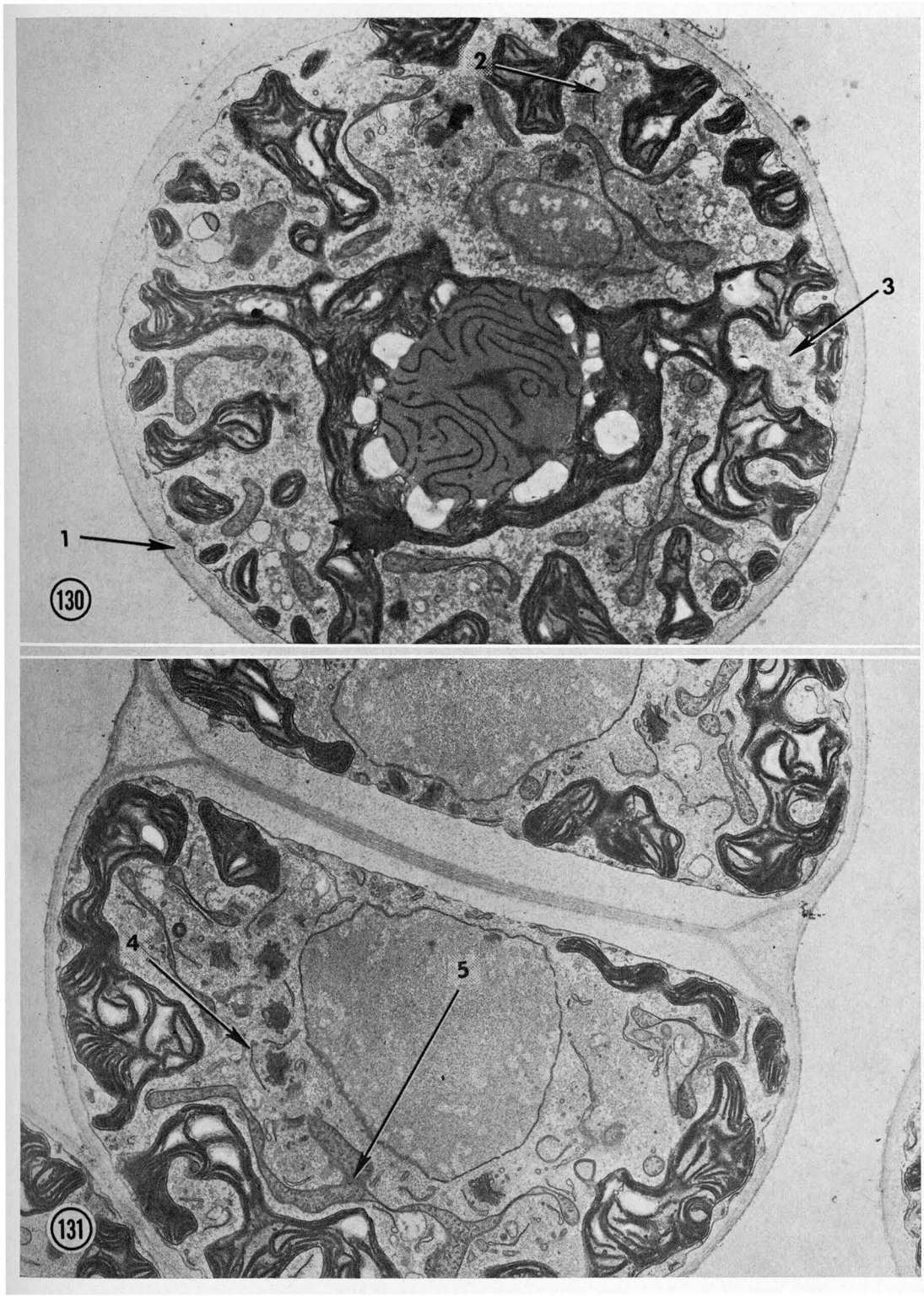
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Fig. 132–134. ***Tetracystis aggregata.*****—Fig. 132. Young tetrad of cells with thin outer walls and thick inner walls of the newly deposited daughter cells (arrow 1). The Golgi cisternae of this species are frequently inflated (arrow 2), ×7,350.—Fig. 133. Portion of vegetative cell showing thick granular layer of the inner cell well (arrow 3), compressed, unbranched mitochondrion (arrow 4), and variable stacking of the chloroplast lamellae (arrow 5), ×16,200.—Fig. 134. Mature tetrad of daughter vegetative cells. Compare walls (arrow 6) with those of the young tetrad (arrow 1, Fig. 132). Note deep, broad, radial fissuring of chloroplast common in this species (arrow 7), ×7,560.**

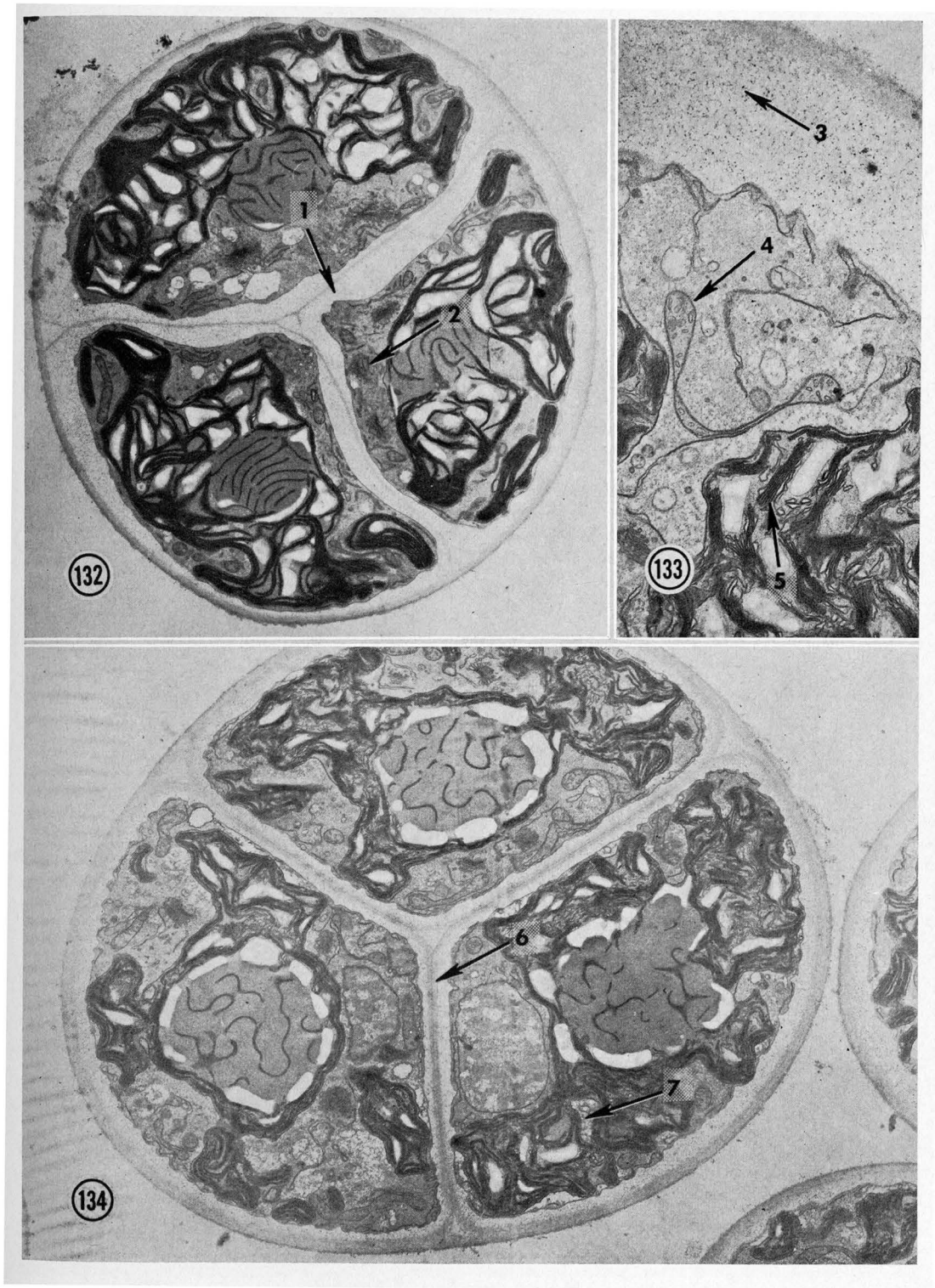
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Fig. 135–137. ***Tetracystis dissociata.*****—Fig. 135. Vegetative cell. Note peripheral fissuring of the chloroplast (arrow 1) and the thick, inner wall layer (arrow 2), ×8,700.—Fig. 136. Portion of vegetative cell showing variable stacking of chloroplast lamellae (arrows 3 and 5). Arrow 4 indicates a tangential section across the chloroplast-limiting membrane, ×16,300.—Fig. 137. Typical ellipsoidal diad stage with Golgi elements (arrow 6) which have indistinct amplexi and characteristically lack cisternal inflation, ×10,550.**

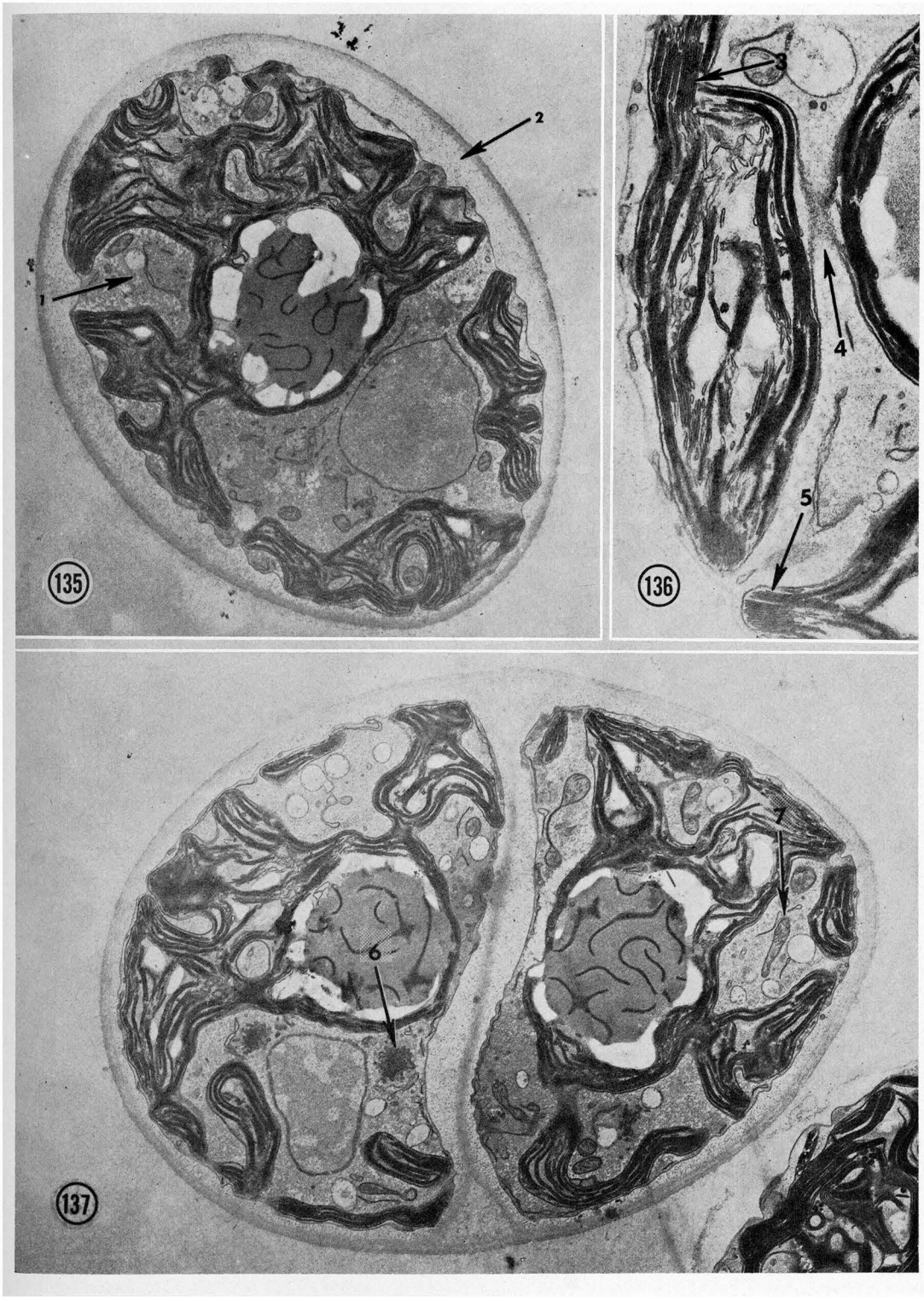
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Fig. 138–140. ***Tetracystis dissociata*** **(continued).—Fig. 138. Mature tetrad of daughter cells which are about to be released by rupture of the weakening electron-dense wall layer of the parent (arrow 2. Arrow 1 denotes a dividing pyrenoid, ×6,750.—Fig. 139. Mature zoosporangium, ×6,300.—Fig. 140. Zoospore with very thin, electron-dense wall layer (arrow 3) and a posterior nucleus (arrow 4). Note parietal chloroplast and equatorial pyrenoid, ×15,700.**

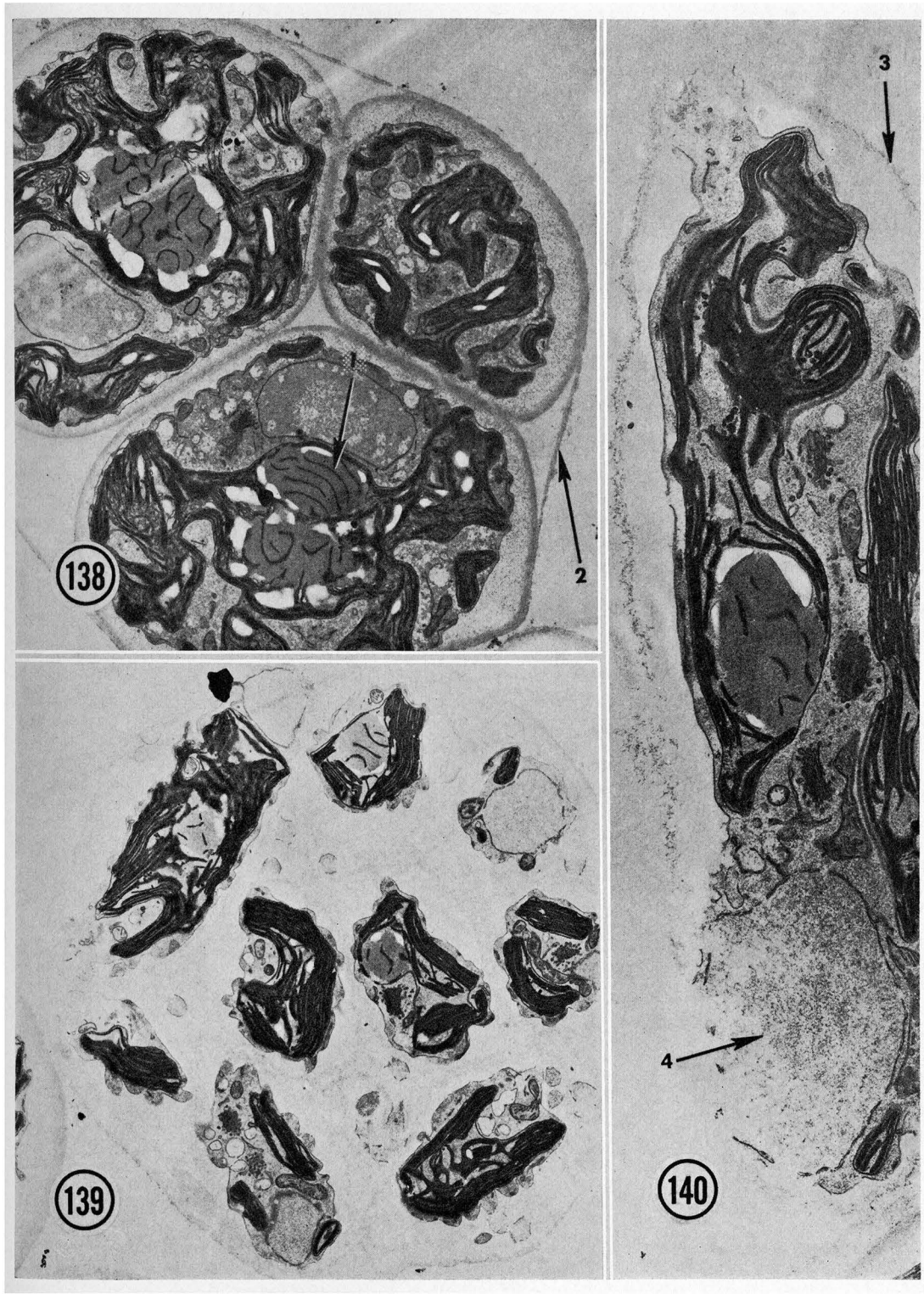
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Fig. 141–142. ***Tetracystis illinoisenis.***—**Fig. 141. Mature vegetative cell with cylindrical mitochondrion (arrow 2); Golgi elements with indistinct amplexi, cisternal elements of which are not inflated (arrow 3); thick inner wall layer (arrow 4); and lack of peripheral invagination (arrow 1) of the thin, parietal choloroplast. Note fissuring, ×8,300.—Fig. 142. Portion of vegetative cell showing stacking of chloroplast lamellae (arrow 5) and pyrenoid to right in photograph, × 22,800.**

Fig. 143. ***Tetracystis excentrica.*** **Mature vegetative cell with marked polar thickening of inner wall layer (arrow 6), lack of peripheral invagination of chloroplast (arrow 7) as well as of fissuring; cylindrical mitochondrion (arrow 8); Golgi elements with distinct amplexus, cisternal elements of the Golgi inflated (arrow 9); and a single, excentric, ellipsodial pyrenoid with 2 starch grains, ×14,500.**

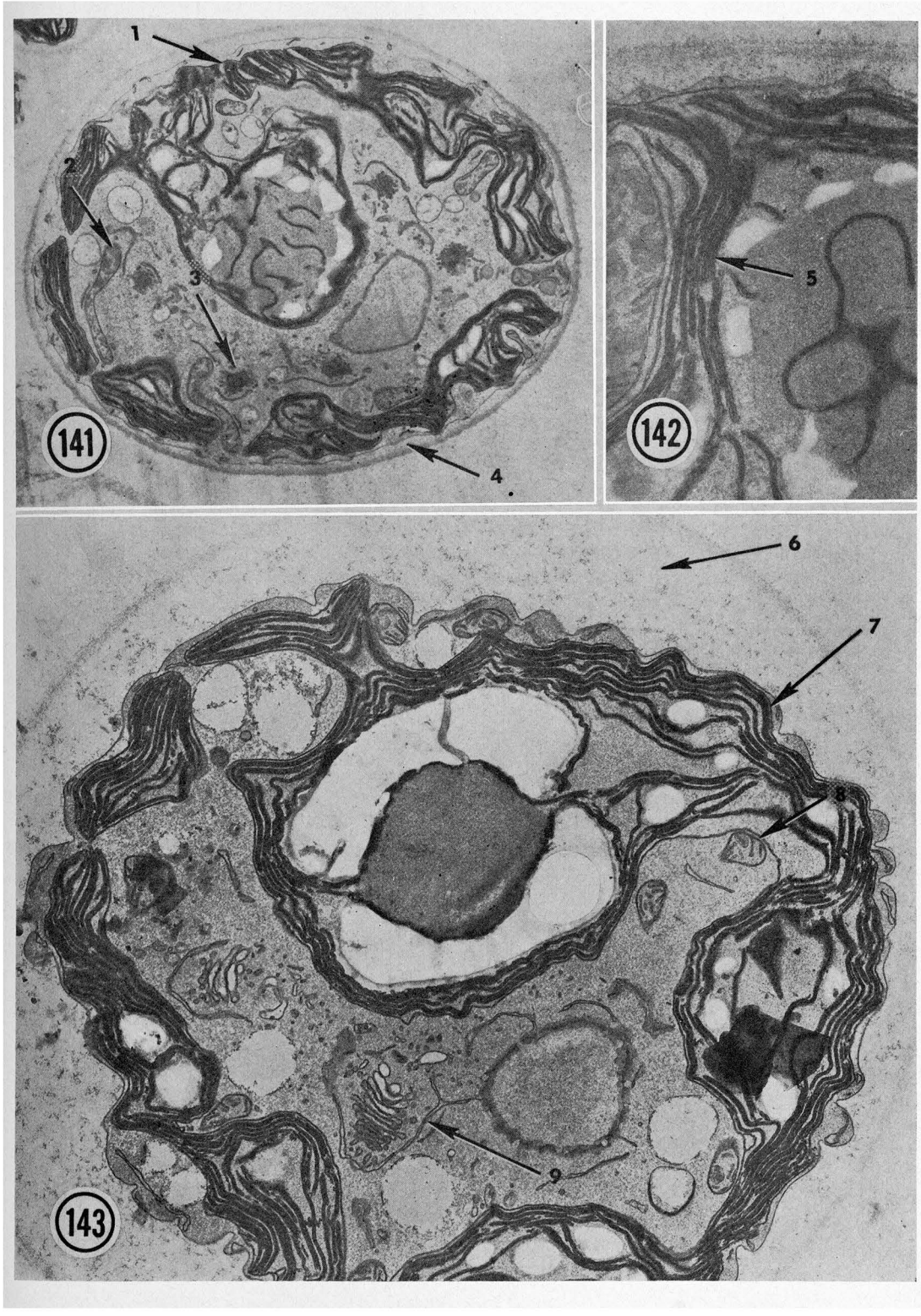
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Fig. 144–146. ***Tetracystis intermedium.*****—Fig. 144. Mature vegetative cell with thick inner wall layer (arrow 2), cylindrical mitochondrion (arrow 1), and chloroplast with few peripheral invaginations and fissures (arrow 3), ×11,700.—Fig. 145. Portion of vegetative cell showing chloroplast with variable lamellar association (arrow 5) and unidentified structure (arrow 4), ×22,800.—Fig. 146. Tetrad of daughter vegetative cells with Golgi elements which have distinct amplexi, cisternal elements of which are inflated (arrow 6). Note granulation in the inner wall layers. ×15,900.**

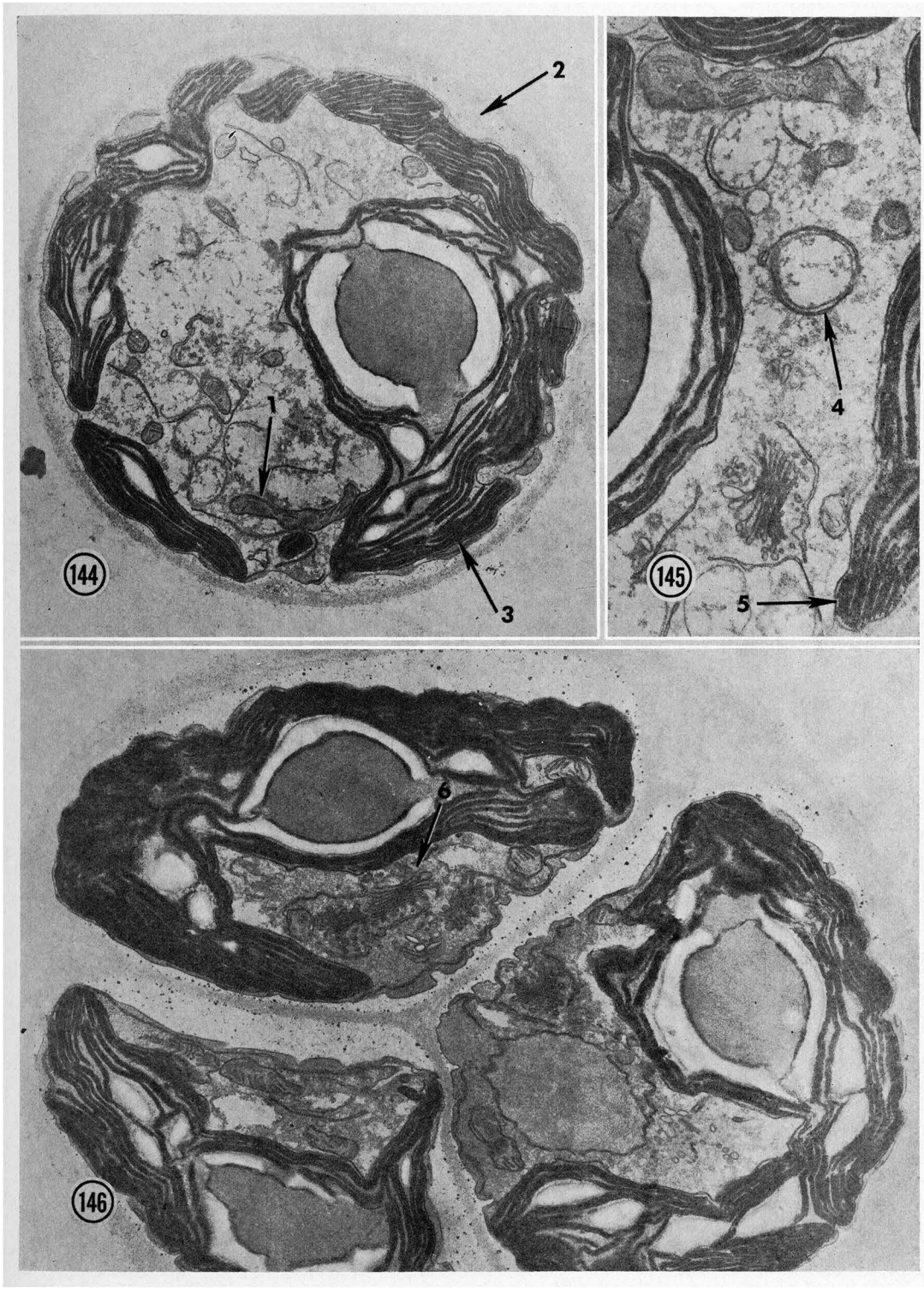
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Fig. 147–150. ***Tetracystis texensis.*****—Fig. 147. Mature vegetative cell with cylindrical mitochondrion (arrow 2), thin outer electron-dense wall (arrow 3), and chloroplast lacking peripheral invaginations (arrow 1), ×9,000.—Fig. 148. Portion of mature daughter vegetative cell showing Golgi apparatus with distinct amplexi, their cisternal elements characteristically inflated, ×14,300.—Fig. 149. Portion of mature vegetative cell showing variable, associated lamellar components of the choloroplast (arrow 4), ×19,000. —Fig. 150. Portion of an octad of daughter vegetative cells, peculiar only to this species. Note the lack of peripheral invagination of the chloroplast (arrow 5), variable thickening of the inner granular wall layers, and the amplexus of the Golgi apparatus with inflated cisternae (arrow 6), ×9,750.**

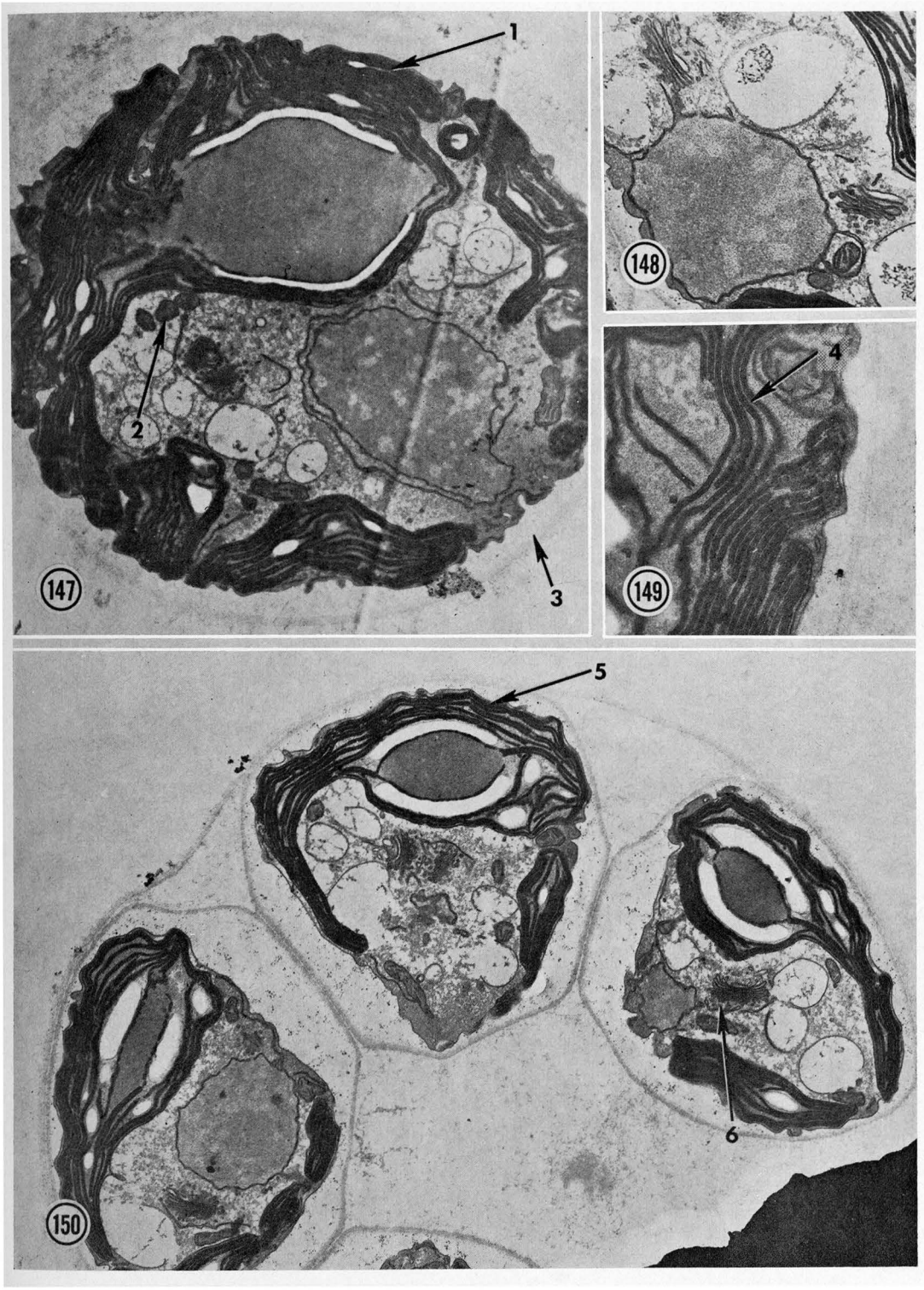
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Fig. 151–152. *Tetracystis pulchra*.—Fig. 151. Mature vegetative cell with ellipsodial pyrenoid surrounded by 2 starch grains. Note chloroplast lamellae with variable stacking (arrow 1) and thickening of the inner wall layers, ×26,400.—Fig. 152. Tetrad of daughter cells showing chloroplast which lacks peripheral invaginations (arrow 3) and which is thin and parietal. Note cylindrical mitochondrion (arrow 5), Golgi apparatus with distinct amplexus, the cisternae characteristically inflated (arrow 4); note also thickened inner wall layer of the daughter cell (arrow 2), ×12,800.

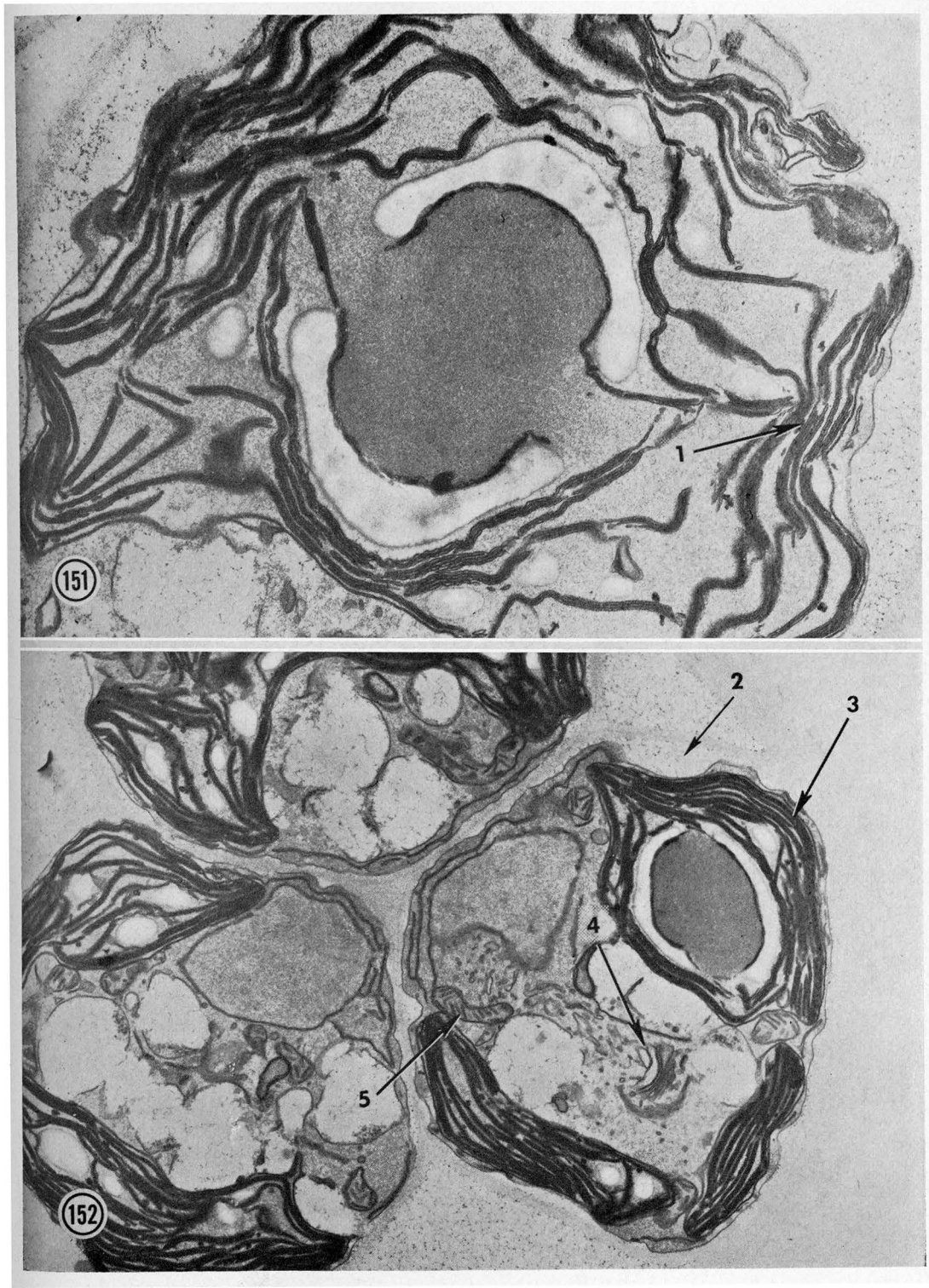
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Fig. 153–155. *Tetracystis aplanosporum*.—Fig. 153. Mature vegetative cell showing thin inner and outer wall layers (arrow 1), cylindrical mitochondrion (arrow 3), and chain of "vacuoles" (arrow 2) which repesent an inflated stack of Golgi cisternae, ×9,200.—Fig. 154. Mature tetrad of daughter cells. Note the loosely associated parent wall (arrow 4) and the Golgi apparati with distinct amplexi and inflated cisternal elements (arrow 5). A contractile vacuole is present just above the nucleus, ×13,600.—Fig. 155. Detail of chloroplast of mature vegetative cell showing stacks of 1–2 lamellar discs alternating irregularly with 3–5 discs (arrow 6). Note the peripheral invagination of the massive chloroplast (arrow 7), ×34,400.

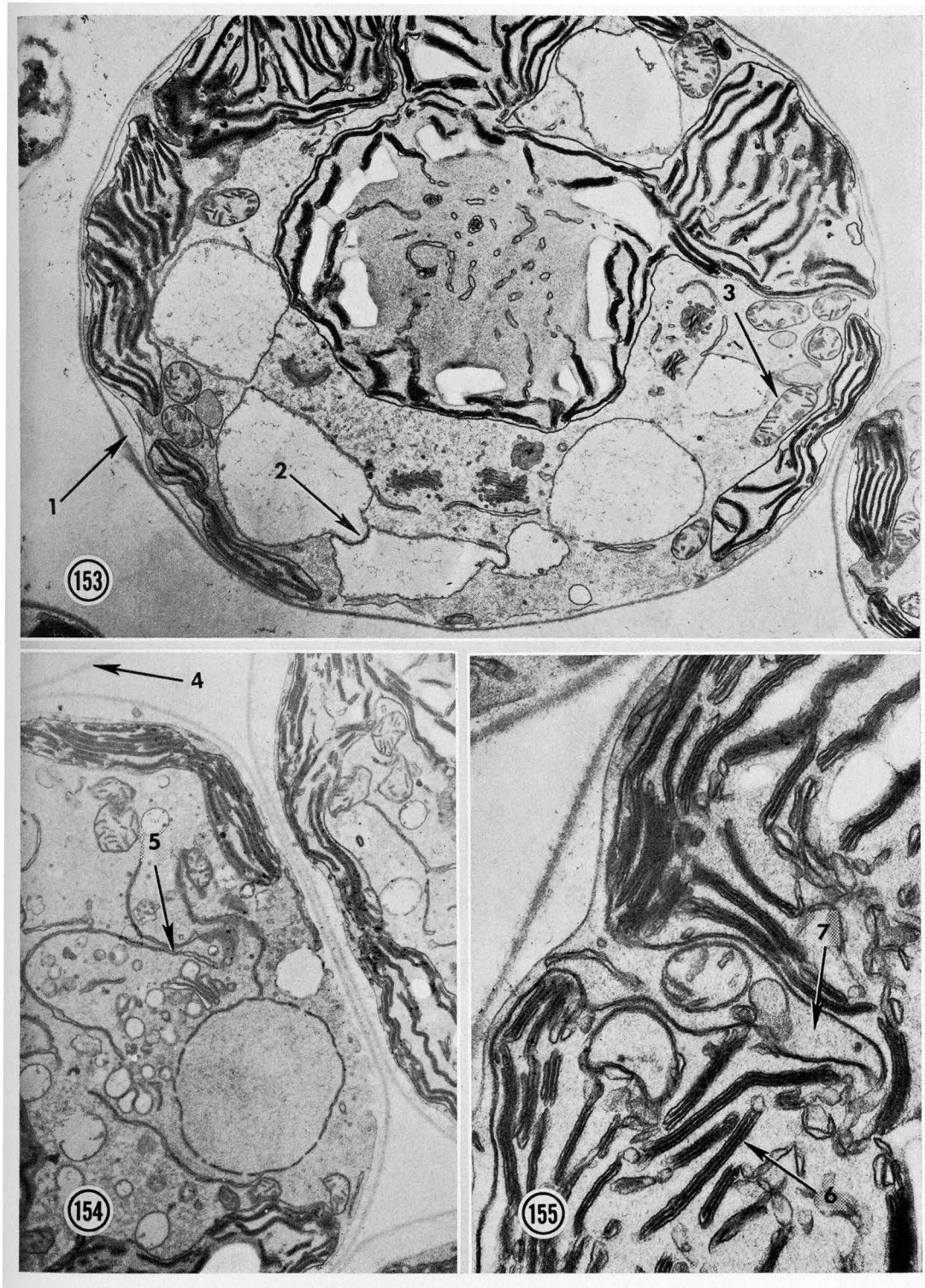
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Fig. 156–158. ***Tetracystis pampae.***—**Fig. 156. Young vegetative cells with cylindrical mitochondrion (arrow 2) and thinner, outer wall layer (arrow 1). Note parietal chloroplast, ×8,250.—Fig. 157. Golgi apparatus with distinct amplexus and cisternal elements which are characteristically inflated, though not shown to a great degree here (arrow 3). Pyrenoid is to right in photograph, ×20,500.—Fig. 158. Mature vegetative cell. Note the triple-disc lamellar system of the chloroplast (arrow 6) and of the pyrenoid (arrow 5). The chloroplast does not have peripheral invaginations (arrow 4), ×16,200.**

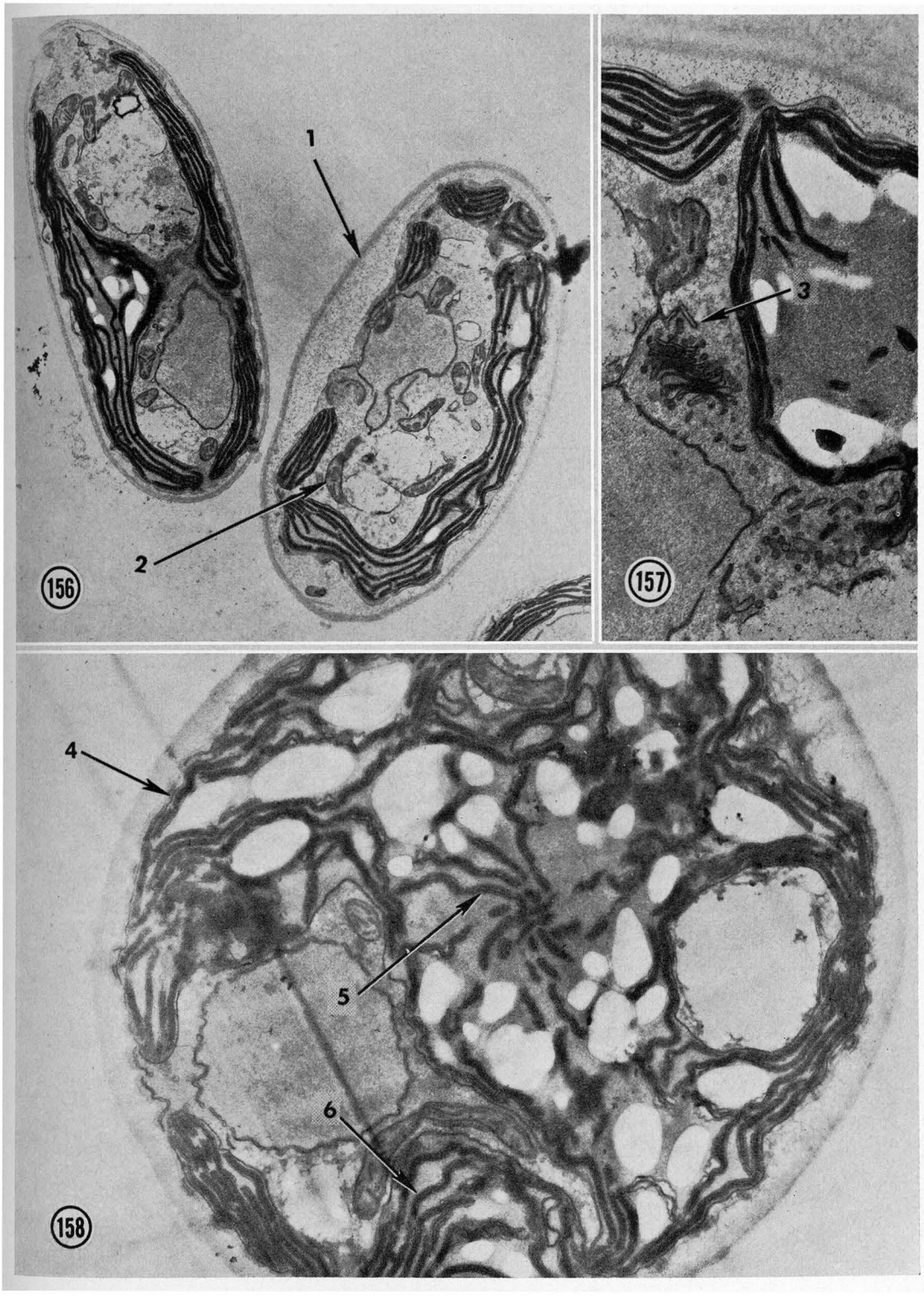
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Fig. 159–162. Pyrenoid types among *Tetracystis*.—Fig. 159. Pyrenoid showing double-disc lamellar system penetrating it (arrow 1). Note parallel orientation of pyrenoid lamellae which is characteristic of this type (*T. isobilateralis*), ×17,800.—Fig. 160. Pyrenoid of *T. aeria* (C–6) showing contorted, double-disc system penetrating it (arrow 2), and characteristic reticulations of the chloroplast which usually lie near the pyrenoid, ×14,200.—Fig. 161. Pyrenoid of *T. pampae* showing tripledisc lamellar system penetrating its matrix (arrow 3), ×21,800.—Fig. 162. Pyrenoid of *T. isobilateralis* showing the flattened or "disc"-like nature of the lamellar components which penetrate it. Note especially the perforations (arrow 4) in thees lamellae which are absent in lamellae situated in the chloroplast proper (arrow 5), ×16,100.

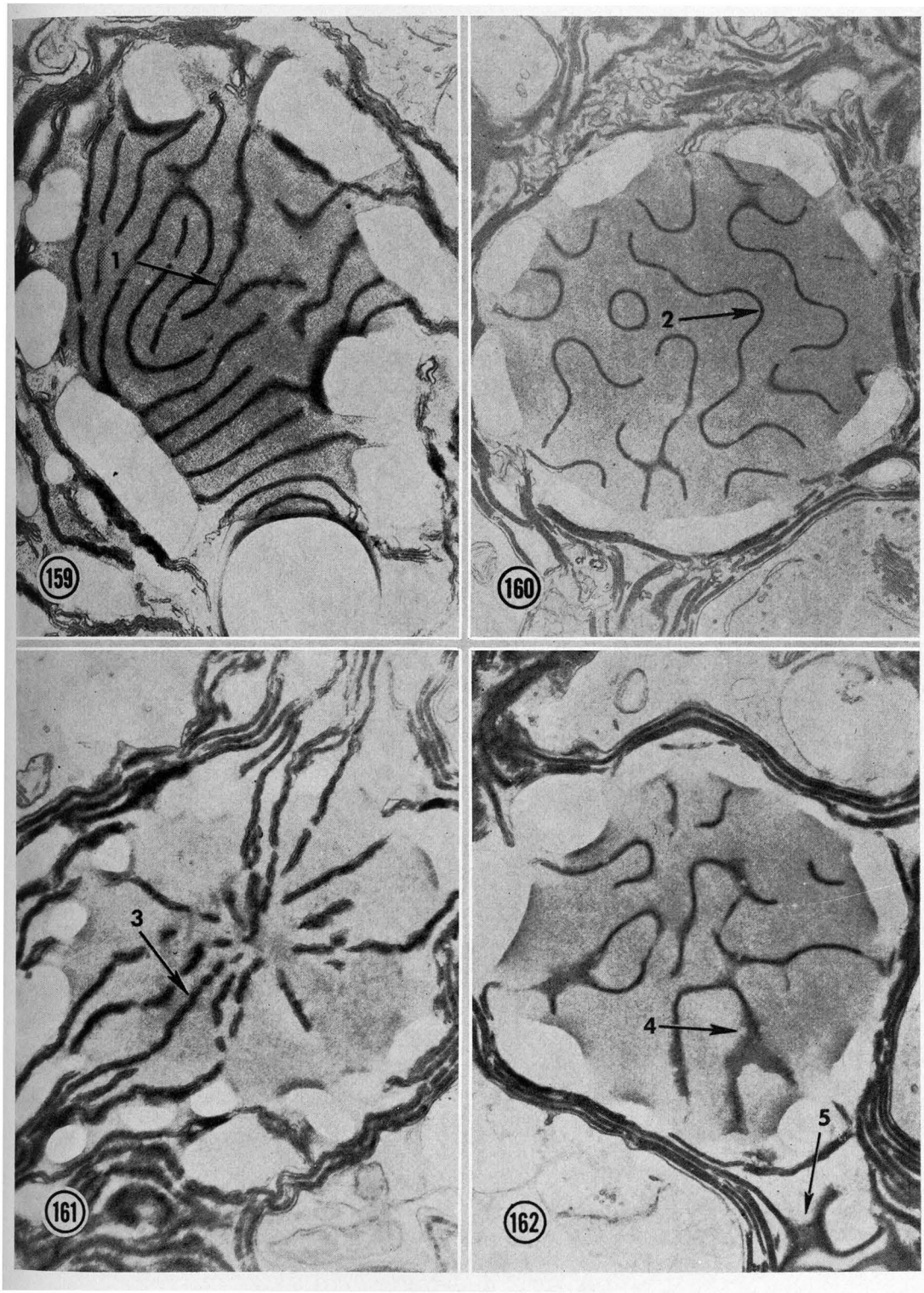
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Fig. 163–166. Pyrenoid types in *Tetracystis* (continued).—Fig. 163. Pyrenoid of *T. texensis* with 2 starch grains (arrows 1 and 2). Note the pyrenoid lamellae which lie internal to the starch (arrow 3). These lamellae become reduced from a double-disc system (arrow 4) to a single, convoluted disc system (arrow 3) at 1 or more loci along the pyrenoid matrix, ×31,000.—Fig. 164. Pyrenoid of *T. aplanosporum* showing single tubular lamellar elements which penetrate the matrix (arrow 5) and which are occasionally associated in pairs (arrow 6), ×23,800.—Fig. 165. Pyrenoid of *Chlorococcum* sp. (tetra isolate) showing tubular elements (arrow 7) which penetrate through a single, surrounding starch grain, ×21,800.—Fig. 166. Pyrenoid of *C. multinucleatum*. Note the lack of starch synthesis on the periphery of the matrix and the presence of reticulated tubules (arrow 9) which penetrate the pyrenoid matrix. Arrow 8 indicates a tubule in cross-section, ×15,600.

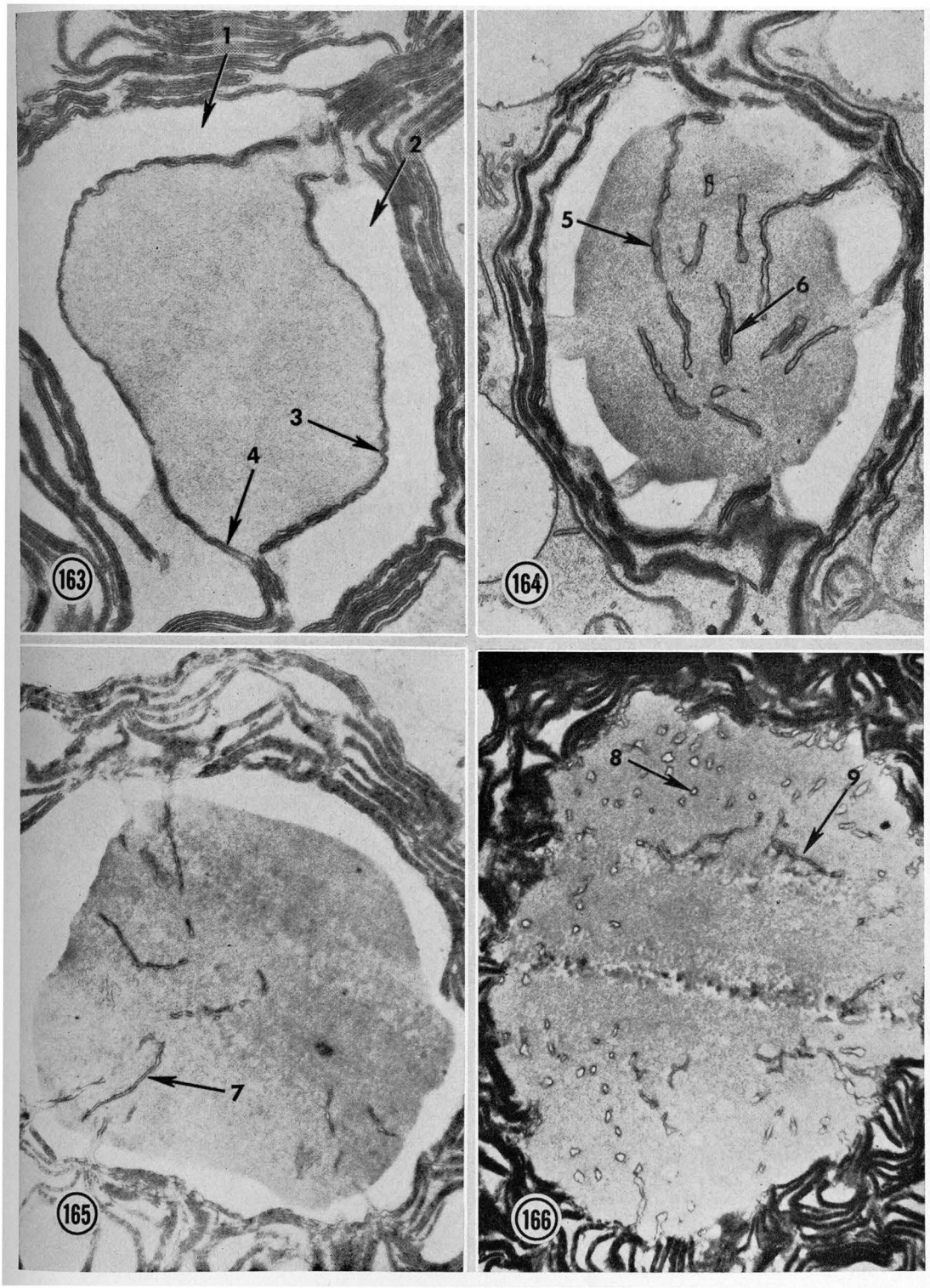
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Fig. 167–170. Stages of pyrenoid division in *Tetracystis isobilateralis*.—Fig. 167. Early stage of pyrenoid division in which the pyrenoid lamellae have become parallel (arrow 3) and have cleaved in a plane (arrow 2) perpendicular to that of the lamellar zone. Note the invaginating pyrenoid lamella (arrow 1), ×16,700.—Fig. 168–169. Different sections through the same pyrenoid at a later stage in division. Note invaginating pyrenoid lamellae in half (arrow 8). Arrows 4, 6, 7, and 9 indicate continuity of parallel pyrenoid lamellae to invaginating pyrenoid lamellae. The chloroplast lamellae are beginning to invaginate at this stage (arrow 5). Fig. 168, ×13,000; Fig. 169, ×14,000.—Fig. 170. Later stage in pyrenoid division in which the chloroplast lamellae (arrow 12) have nearly cleaved the pyrenoid (arrows 13 and 14). Note that the pyrenoid lamellae are still parallel (arrow 15) and that the invaginating chloroplast lamellae appear to split the pyrenoid lamellae (arrow 10). Arrow 11 indicates an unknown structure, ×15,000.

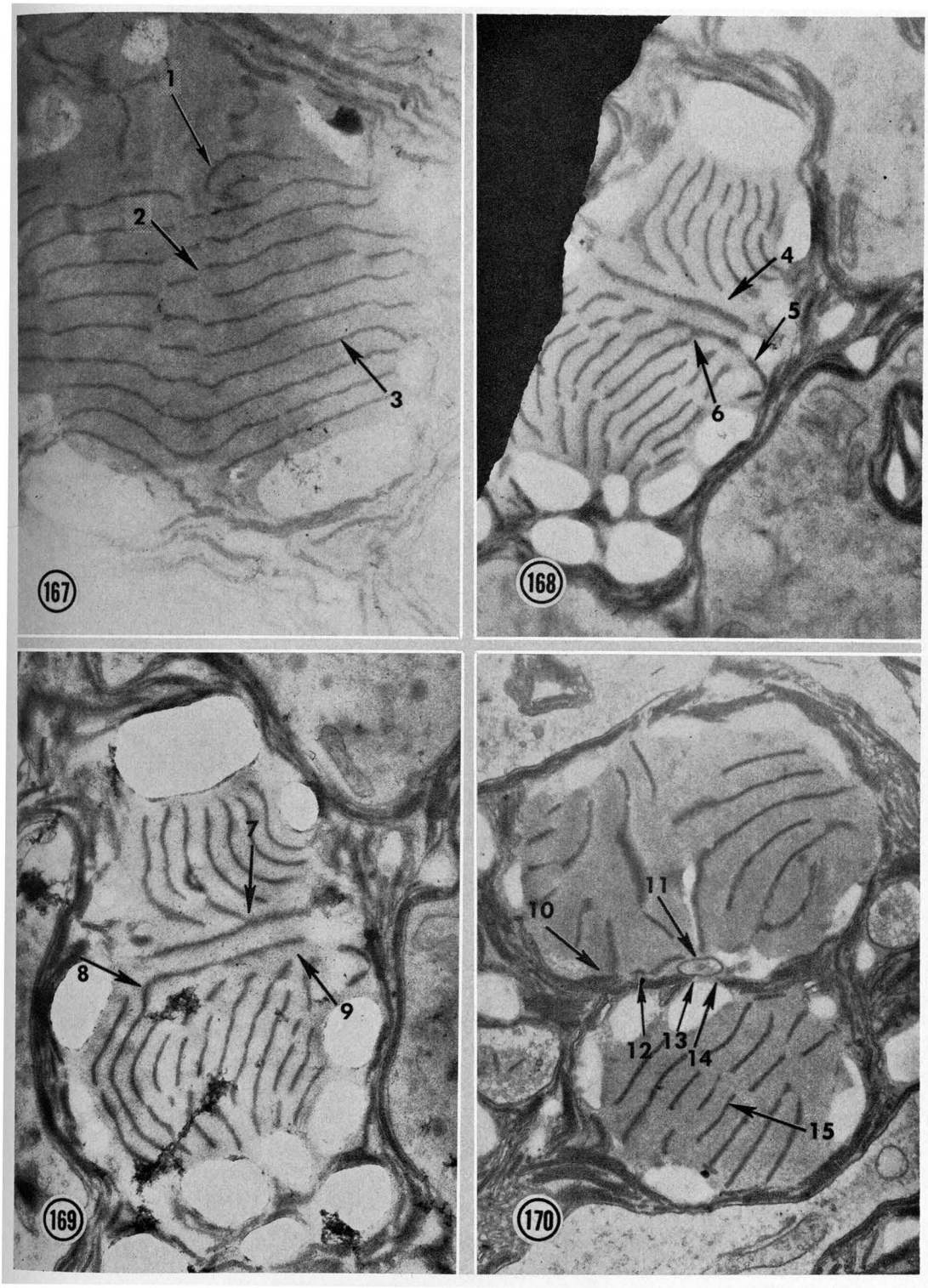
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Fig. 171–172. Pyrenoid division in *Tetracystis isobilateralis* (continued).—Fig. 171. Later stage of pyrenoid divison in which the chloroplast lamellae have completely split the pyrenoids in half (arrow 2). Note starch synthesis which is now becoming active on the interfaces of the daughter pyrenoids (arrow 1). Note the persistence of parallel lamellae of the daughter pyrenoids (arrow 3), ×17,000.—Fig. 172. Final stage in pyrenoid division in which the separated pyrenoid matrices have become discernible (arrow 5), and in which the pyrenoid lamellae have re-oriented (arrow 6). Note also that the invaginating chloroplast lamellae are more profuse (arrow 4). Pre-fixed in glutaraldehyde, then post-treated with 2% $LiMnO_4$, ×23,600.

Fig. 173. Pyrenoid division during vegetative cell division in *Tetracystis aplanosporum* in which the chloroplast-limiting membrane splits the pyrenoid matrix (arrow 8) and the pyrenoid tubules (arrow 7), ×15,600.

Fig. 174. Pyrenoid division of *Tetracystis aeria* (C–6) in which the chloroplast-limiting membrane separated the pyrenoids (arrow 10). Note the probable splitting of the pyrenoid lamellar discs (arrow 7) in the division zone, ×19,000.

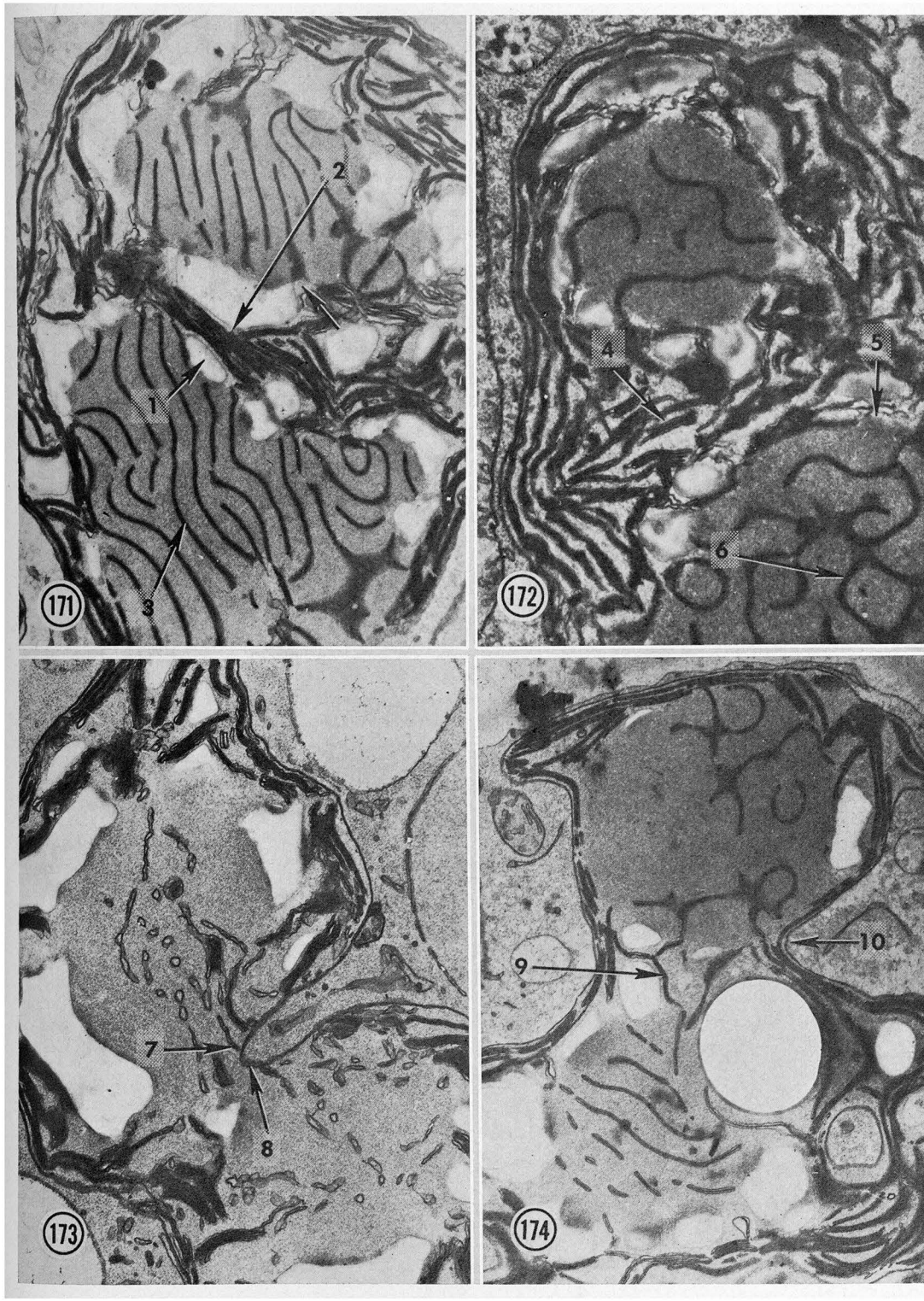
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Fig. 175–179. Formation of Golgi cisternae in *Tetracystis*.—Fig. 175. Amplexus (arrows 1 and 2) with multiple Golgi systems in *T. texensis*, × 25,000.—Fig. 176. Amplexus system of *T. excentrica*. Note the close proximity of the several Golgi apparatus to the nucleus in which anticlinical sections of the amplexi show a characteristic spur (arrow 3) and an area along the amplexus which appears to be budding to form the cisternal elements of the Golgi (arrow 4), ×18,000.—Fig. 177. Tangential section through a Golgi amplexus system of *T. texensis* showing budding elements (arrows 4, 5 and 6) of the amplexus and tubular hexagonal network on the periphery of the Golgi (arrow 8) elements, ×40,000.—Fig. 178. Golgi apparatus of *T. texensis* surrounded by budding amplexus (arrows 9, 10). Arrows 12 and 13 show a continuity between the amplexus and the Golgi cisternae. Arrow 14 shows a portion of the amplexus which extends to make contact with the nucleus (out of picture, below), ×39,000.—Fig. 179. Budding amplexus system of *T. excentrica* showing attachment to nucleus (arrow 16 indicating nuclear pore) and proliferation of lamellar elements of the amplexus in close proximity to the Golgi cisternae. Note that the Golgi cisternae nearest the encompassing portion of the amplexus are always non-inflated and more closely packed than those away from the amplexus, ×32,500.

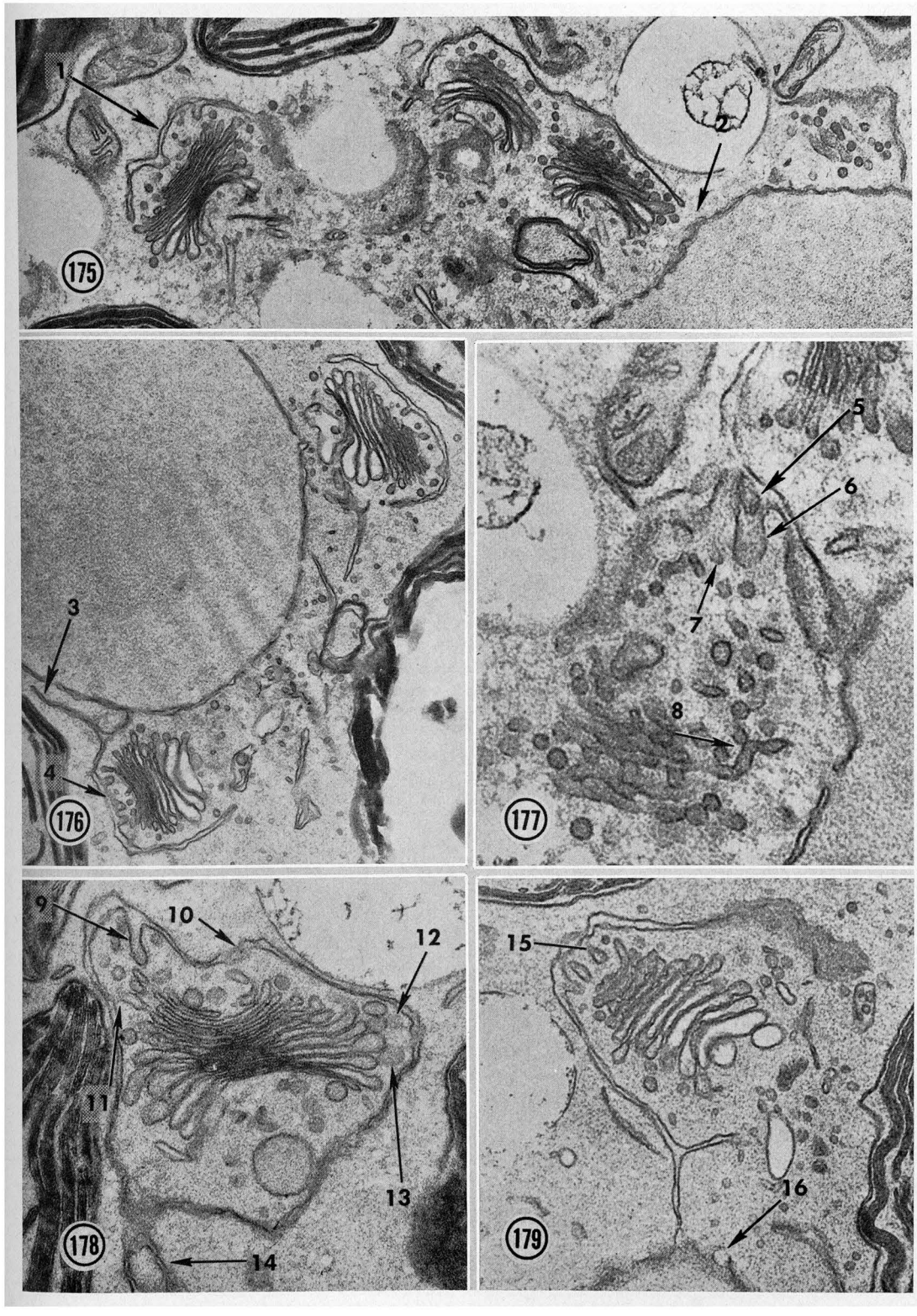
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Fig. 180–185. Possible vacuole development from Golgi cisternae in *Tetracystis*.—Fig. 180. Various stages of cisternal inflation (arrows 1, 2, and 3) in *T. aplanosporum*. Arrow 4 indicates a contractile vacuole, ×16,400.—Fig. 181. Probable greatly inflated cisternal elements which were deposited in pairs. (*T. aplanosporum*) (arrow 6). Arrow 5 shows what may be adjacent cisternal membranes which remained together as the cisternae were deposited in the cytoplasm, ×15,400.—Fig. 182. Inflated cisternal elements (arrows 8, 9) of *T. aplanosporum* (fixed in OsO_4). Arrow 9 shows uninflated cisternae nearest the amplexus, ×31,500.—Fig. 183. Inflated Golgi elements of *T. excentrica* showing undirectional inflation always opposite the encompassing amplexus, ×34,100.—Fig. 184. Tangential section of Golgi apparatus with inflated cisternae (arrow 10) and uninflated portion of another Golgi apparatus (arrow 11), ×26,900. —Fig. 185. Golgi apparati of *T. excentrica* post-stained wtih 5% of $Ba(MnO_4)_2$. Note the starch (arrow 12) which was densely stained by this process; the inflated Golgi cisternae (arrow 13) were not so stained, this indicating that a soluble product forming the vacuolar elements was probably leeched from the Golgi during the fixation process, ×22,800.

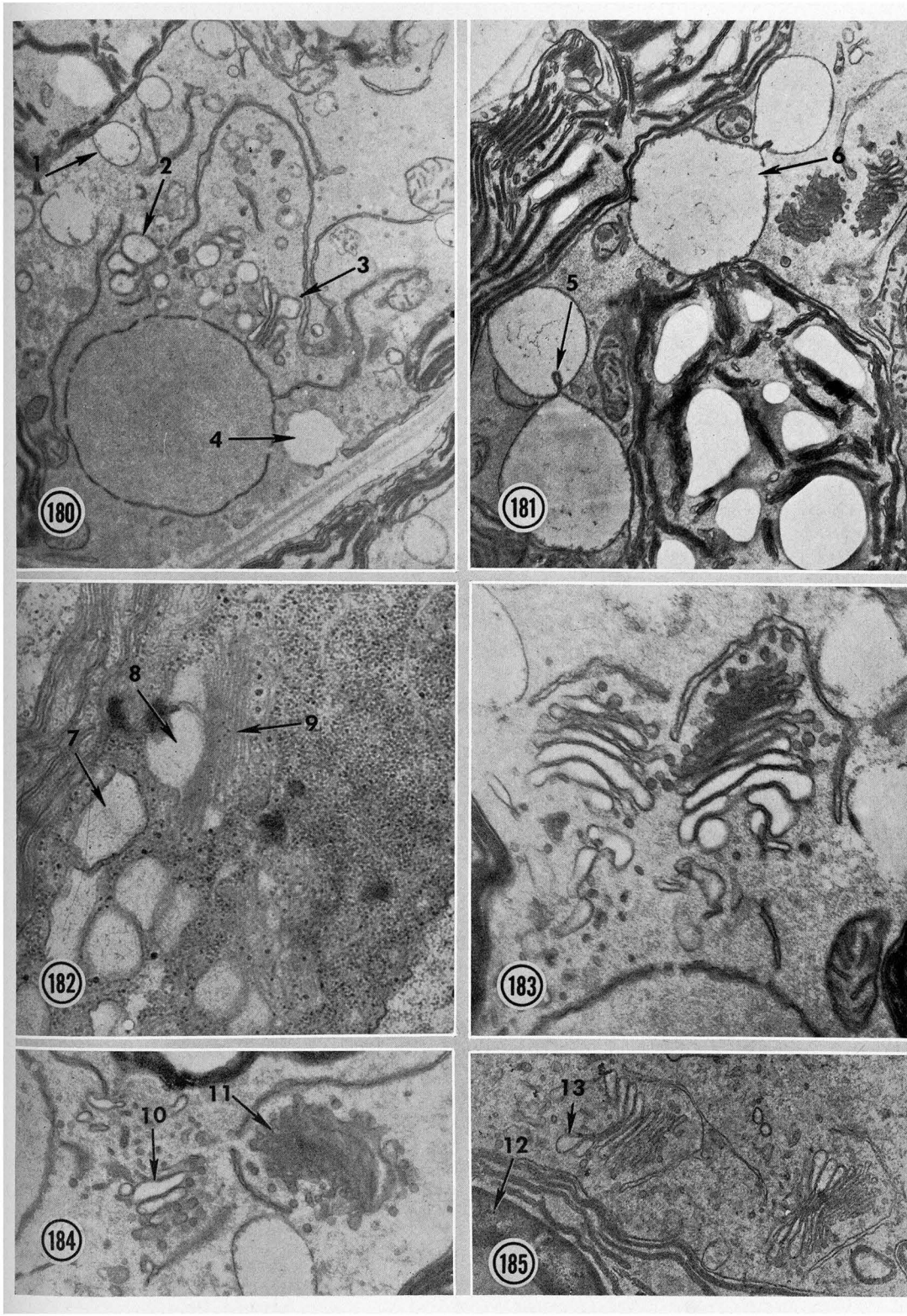
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Fig. 186–189. Contractile vacuoles in *Tetracystis aplanosporum*.—Fig. 186. One contractile vacuole completely closed (arrow 1). Note the radial, tubular elements of the vacuolar membrane. The other contractile vacuole is completely open (arrow 2). Note the granular strings in the vacuole itself, ×24,100.—Fig. 187. Partially closed contractile vacuole (arrow 5) with associated endoplasmic reticulum (arrow 4). Note the absence of radial proliferations on the peripheral side of the vacuole (arrow 5), ×20,900.—Fig. 188. Contractile vacuole caught in the process of contracting (arrow 6). Arrow 7 indicates associated endoplasmic reticulum. Arrow 8 shows a tangential section through a closed contractile vacuole, ×27,900.—Fig. 189. Contractile vacuole of *T. aplanosporum* fixed with 1 % OsO_4 (arrow 9). Note lack of ribosomes in close proximity to the radial proliferations of the vacuolar membrane, ×58,000.

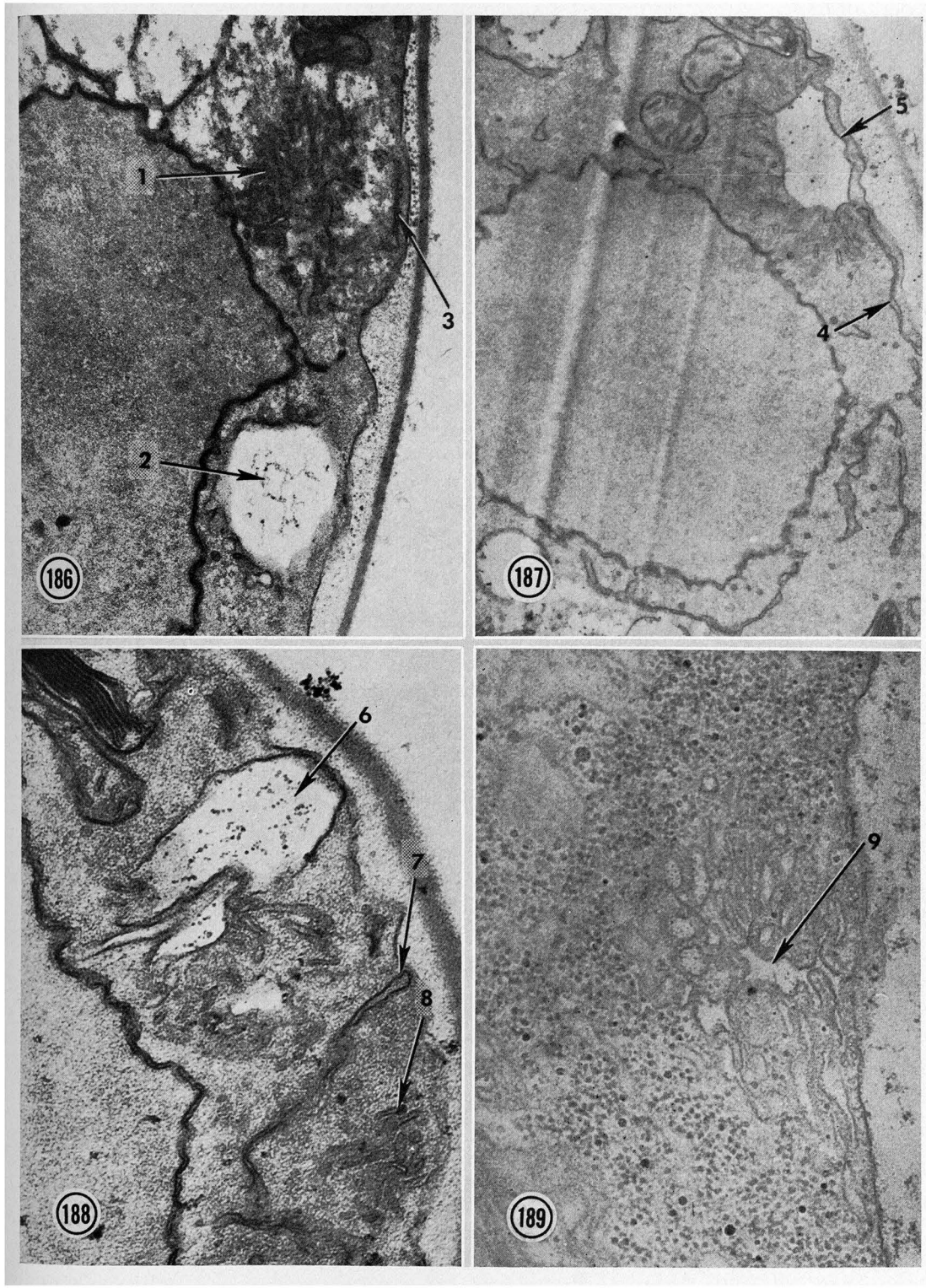
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Fig. 190–192. Vegetative cell division in *Tetracystis aggregata*.—Fig. 190. Mature vegetative cell undergoing furrow formation (arrow 2). Note the close proximity of the endoplasmic reticulum and Golgi apparatus (arrow 1) to the advancing cleavage furrow (arrow 2), ×13,500.—Fig. 191. Portion of cleavage furrow which has cut through most of the cell. Note the "intercellular space" (arrow 4) and the zone which separates the fine granulation from the coarse granulated inner-wall layer of the parent cell (arrow 3). Note also that inner wall deposition (as evidenced by fine granulation) has not occurred in the area of the cleavage furrow at the bottom of the photograph, ×16,500.—Fig. 192. Later stage of the same area when cell division has been completed. Note the presence of coarse granulation in the inner layers common to the parent and daughter cell. Note the electron-dense wall layer deposited around the daughter cells (arrow 7) which is contiguous with the electron-dense layer of the parent cell (arrow 6). Note the absence of coarse granulation (arrow 5) in the intercellular space which appears to be blocked by the electron-dense walls of the daughter cells. When such coarse granulation is absent, no further deposition of the perent electron-dense wall occurs, and this layer deteriorates (arrow 6), ×22,800.

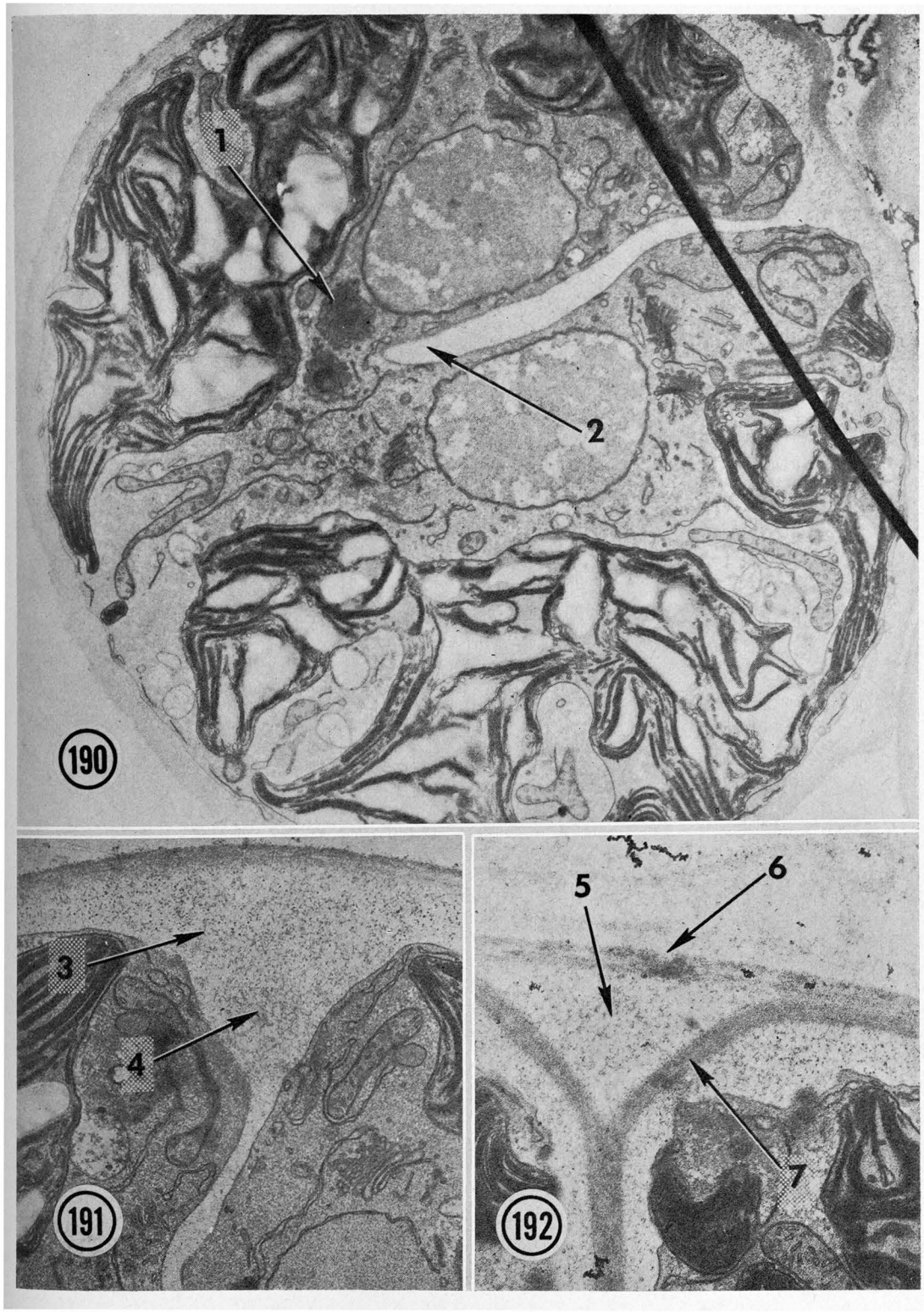
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Fig. 193–194. Vegetative cell division in *Tetracystis isobilateralis*.—Fig. 193. Diad undergoing vegetative cell division to form a triad of daughter cells. Electron-dense wall layer is deposited immediately behind advancing cleavage furrow (arrow 1). Arrow 3 shows intercellular space devoid of coarse granulation. Arrow 5 indicates probably origin of coarse granulation from vacuoles in the cytoplasm. The granules appear to migrate through the cytoplasm (arrow 2) to the area of wall formation and thence into the inner wall layers to form the electron-dense wall layer (arrow 4), ×11,400.—Fig. 194. Detail of cytoplasmic localization of coarse granulation (arrow 6) near node of advancing cleavage furrow, ×23,600.


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Fig. 195–196. Two sections through same cleavage furrow of *Tetracystis aggregata* showing the Golgi apparatus in section (arrow 1) and out of section (arrow 2), ×14,150.—Fig. 196, ×17,600.

Fig. 197. Early vegetative cell division in *T. texensis* showing initiation of cleavage furrows (arrows 4, 5) between divided chloroplasts and zone of vacuolization (arrow 4) between the nuclei. Note the close association of outer nuclear envelopes (arrow 4) ×14,200.

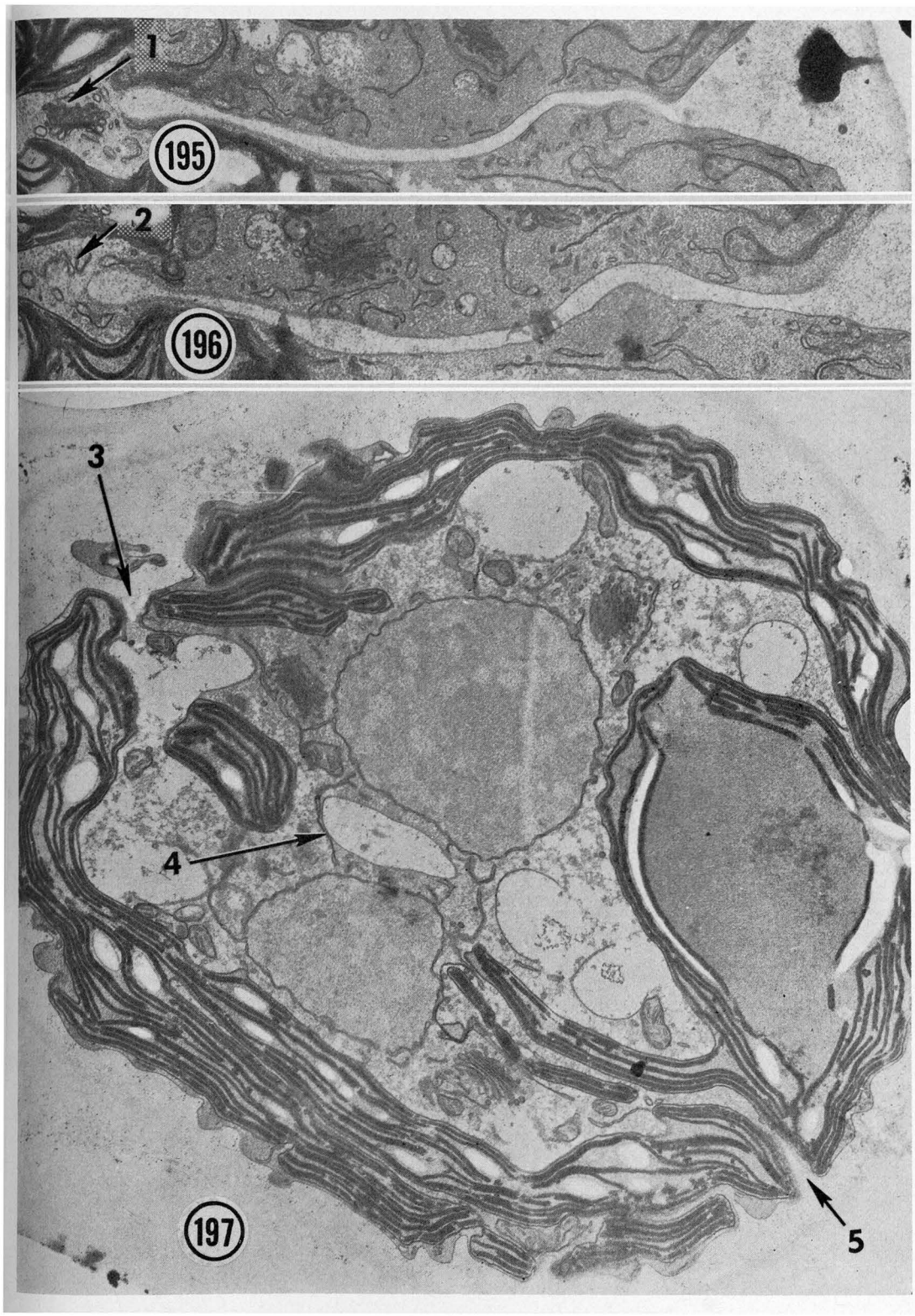
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Fig. 198–199. Later stages of vegetative cell division in *T. texensis* (continued).—Fig. 198. Cleavage furrows (arrows 1, 3) progressing centripetally. Note chromatin of newly formed interphase nucleus (arrow 2), ×13,600.—Fig. 199. Cleavage complete, the furrows having cut around some cytoplasm (arrow 5) between the nuclei. Associated nuclear envelopes appear to have been cleaved by the furrows (arrows 4, 6), ×12,300.

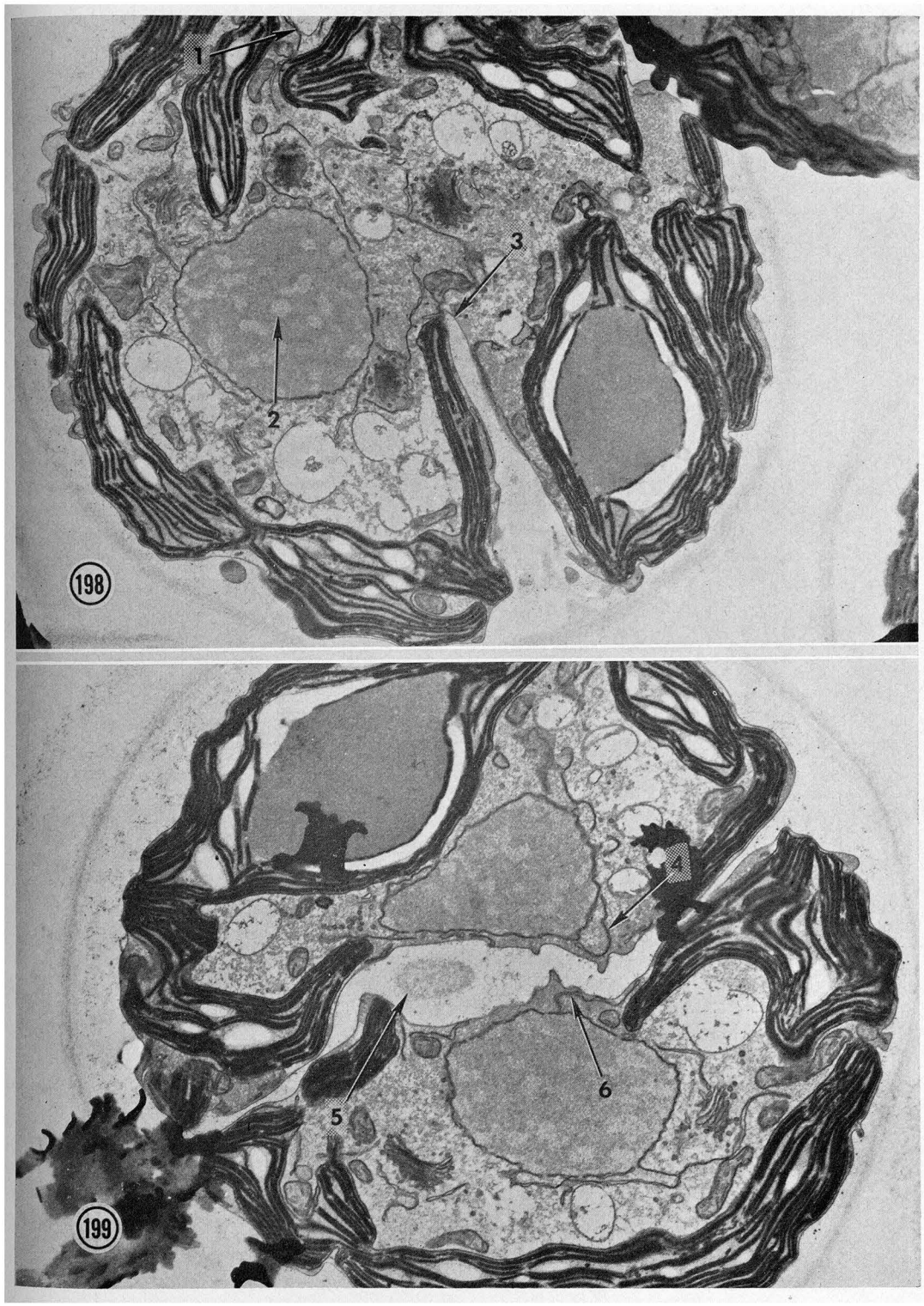
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Fig. 200. Vegetative cell division in *T. excentrica*. Bilateral cleavage furrows have almost cut through cell (arrow 4). Arrow 1 indicates the outer, electron-dense layer of parent wall. Note the increased activity of the Golgi apparati, 4 of which are shown encompassed by 1 amplexus (arrow 2). Tangential section of nucleus (arrow 3), ×10,000.

Fig. 201. Vegetative cell division in *T. aeria* (C–6). Arrows 5 and 6 show possible pathways the cleavage furrows first take, followed by centrifugal deposition (arrows 7, 8), ×6,000.

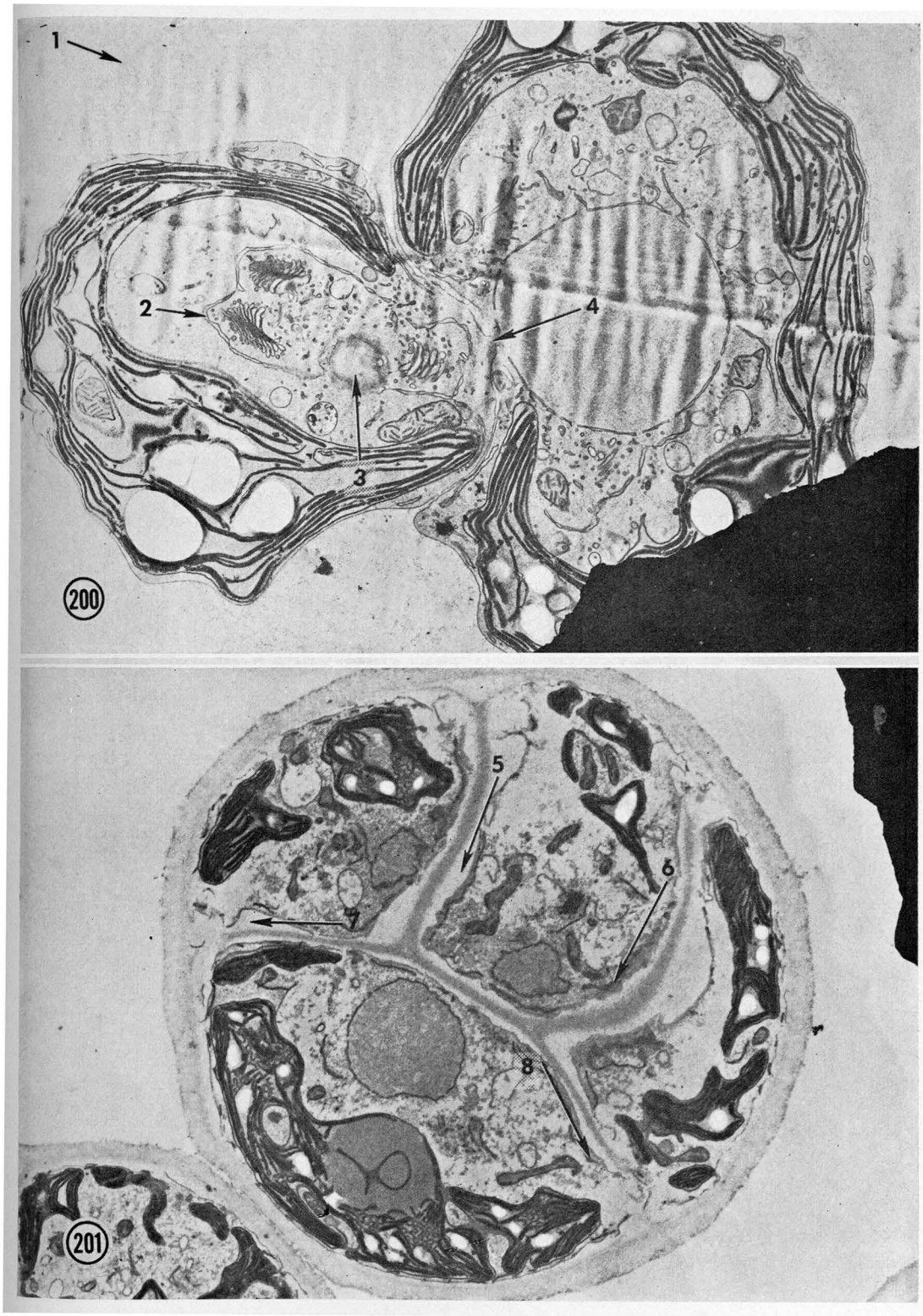
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Fig. 202–205. Early zoosporogensis in *Tetracystis aplanosporum*.—Fig. 202. Early chloroplast cleavage (all arrows 1) in pyrenoid region, as exemplified by the tubular elements of the pyrenoid matrix (all arrows 2). Note the increased activity of the Golgi elements (arrow 3) and the chloroplast lamellar proliferation and increase in chloroplast starch (arrow 4), ×12,700.—Fig. 203. Another early stage of zoosporogenesis in which the pyrenoid, as indicated by the presence of pyrenoid tubules (arrows 2), is being cleaved by the dividing chloroplast at a number of loci (arrows 1), ×12,500.—Fig. 204. A still later stage of zoosporogenesis. Note the absence of starch synthesis on pyrenoid surfaces. Pyrenoid tubules clearly delimit pyrenoid region (arrows 2) Golgi cisternae are greatly inflated during this stage of zoosporogenesis (arrow 3), ×15,200.—Fig. 205. Zoosporogenesis near the completion of pyrenoid fragmentation, yet the pyrenoids can still be discerned by the presence of their tubules (arrows 2) even though they are inactive in starch synthesis. Arrow 1 shows the terminating phase of chloroplast cleavage, ×12,500.

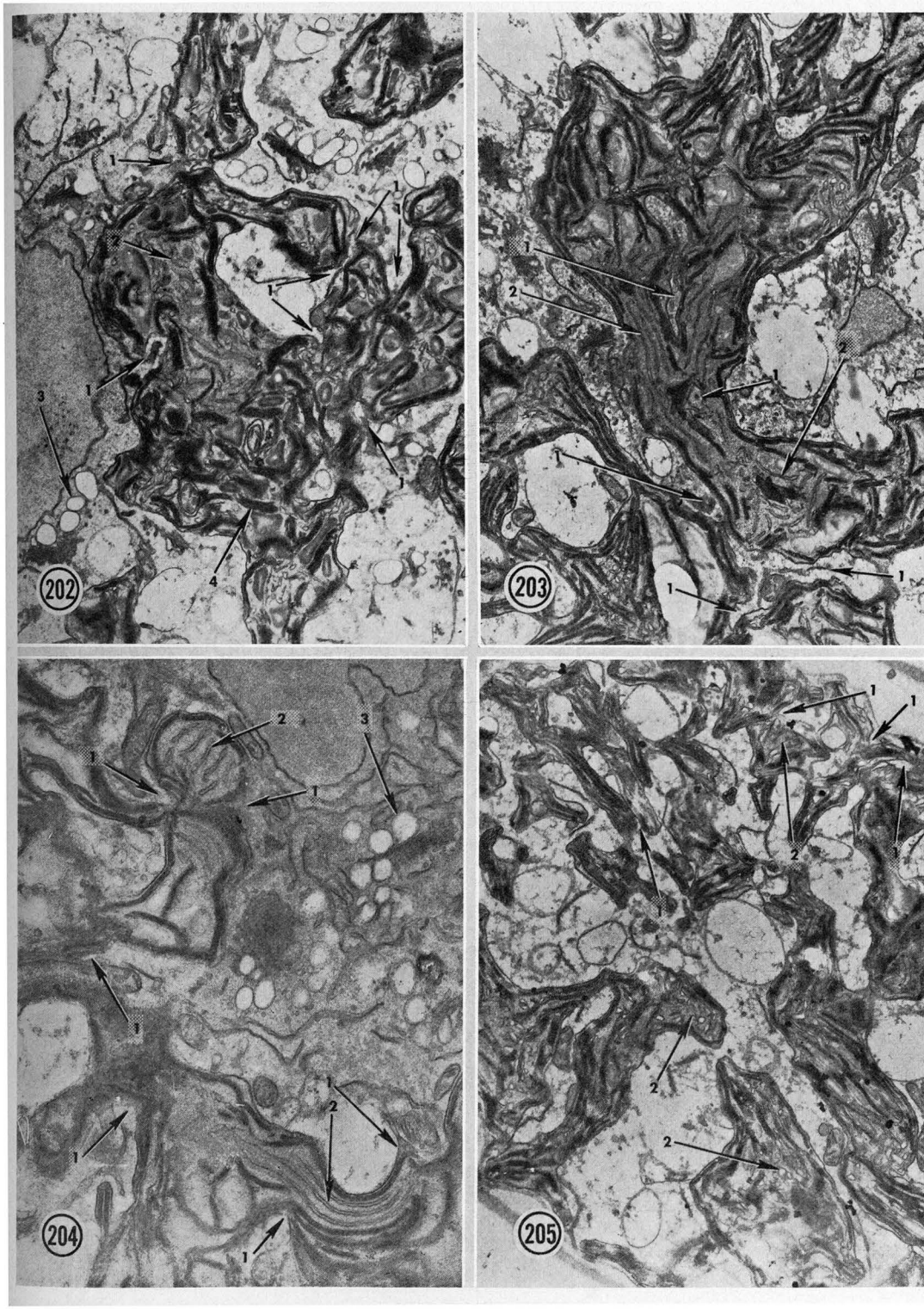
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Fig. 206–207. Cell wall formation during zoosporogenesis.—Fig. 206. Early wall development in *Chlorococcum* sp. (tetra). Note common inner wall layer (arrow 1), endoplasmic reticulum (arrow 2), and splitting of common wall layer (arrow 3), ×18,000.—Fig. 207. Later stage of wall development in *Chlorococcum multinucleatum*. Note endoplasmic reticulum (arrow 6), plasma membrane (arrow 7), electron-dense wall layer (arrow 8), and splitting of common wall layer (arrow 8). Note also that even before wall formation is complete, the flagella (arrow 5) and stigma (arrow 4) are completely developed, ×16,300.

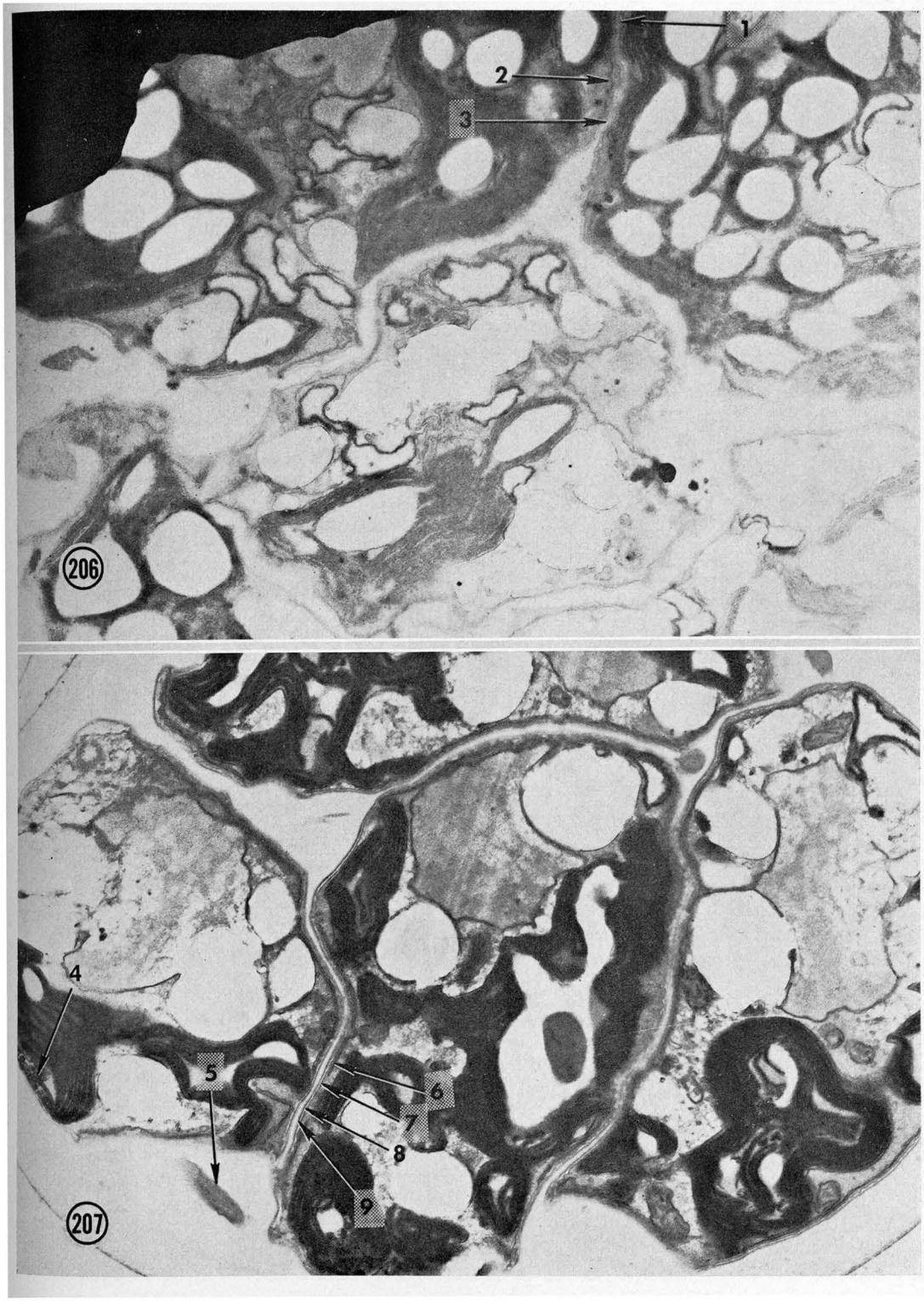
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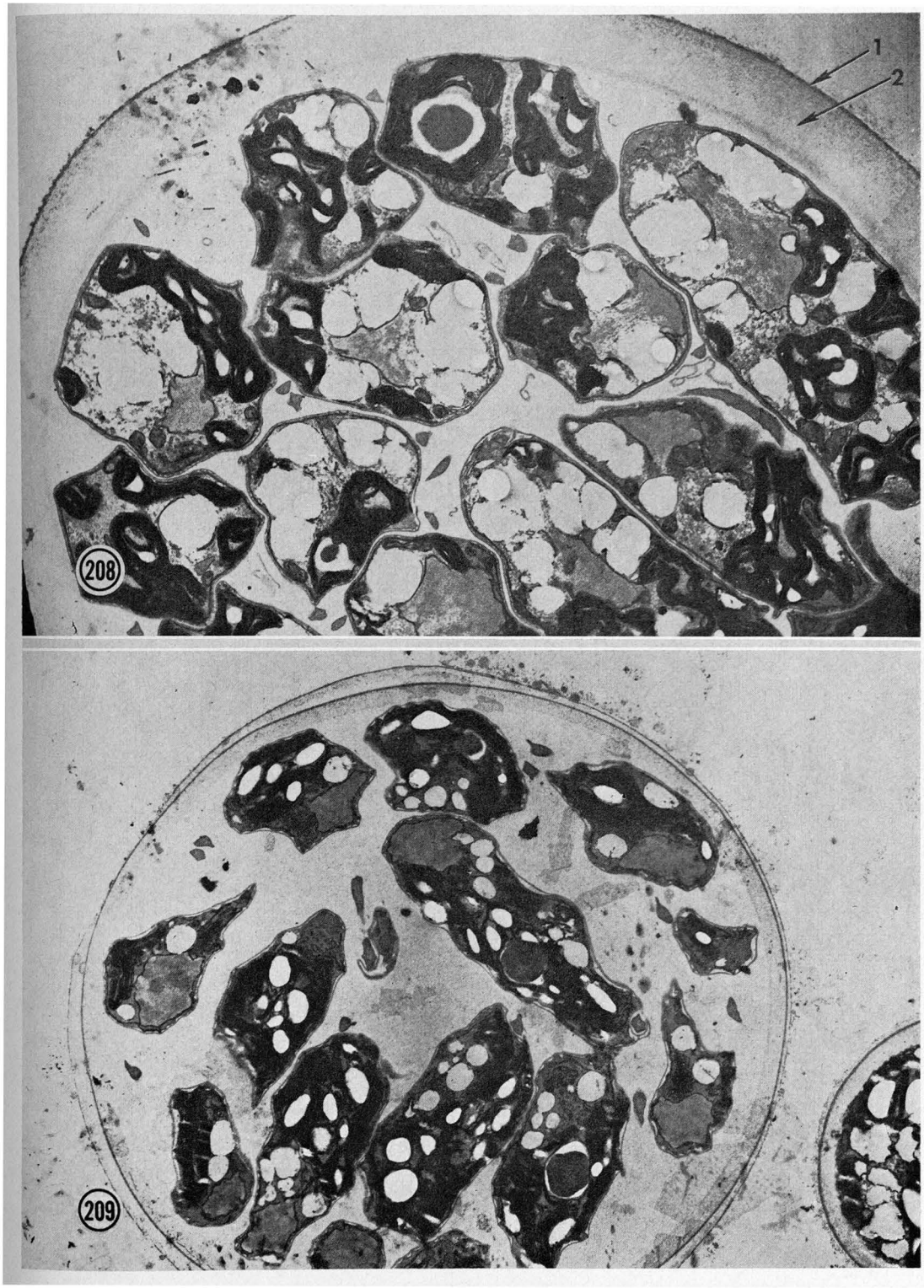
Fig. 208–209. Late zoosporogenesis.—Fig. 208. *Chlorococcum multinucleatum*. Note the separation of zoospore wall from both the outer, electron-dense layer (arrow 1) and inner wall layer (arrow 2) of parent cell, ×9,350.—Fig. 209. *Chlorococcum* sp. (tetra). Late zoosporogenesis, just prior to zoospore release, ×6,000.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Code to all antisera and antigens used in the immunochemical study and which appear in the illustrations to follow:

ANTISERA

A = anti-*Tetracystis aeria* (C–6)
B = anti-*Chlorococcum perforatum*
C = anti-*Tetracystis isobilateralis*
D = anti-*Tetracystis aplanosporum*
E = anti-*Chlorococcum* sp. (tetra isolate)

ANTIGENS:

1 = *T. aeria* (C–6)
2 = *T. aeria* (Pa–3)
3 = *T. dissociata*
4 = *T. isobilateralis*
5 = *T. aggregata*
6 = *T. illinoisensis*
7 = *T. aplanosporum*
8 = *T. pampae*
9 = *T. intermedium*
10 = *T. excentrica*
11 = *T. texensis*
12 = *T. pulchra*
13 = *T. tetrasporum*
14 = C. *perforatum*
15 = C. *diplobionticum*
16 = C. *echinozygotum*
17 = C. *ellipsoideum*
18 = C. *hypnosporum*
19 = C. *macrostigmatum*
20 = C. *minutum*
21 = C. *multinucleatum*
22 = C. sp. (tetra isolate)
23 = C. *oleofaciens*
24 = C. *scabellum*
25 = C. *pinguidium*
26 = C. *punctatum*
27 = C. *vacuolatum*
28 = C. *wimmeri*

Fig. 210–213. Experiments establishing proper conditions for reaction of algal antigens upon double-diffusion and immunoelectrophoresis.—Fig. 210. Experiment to determine the best cup-to-trough distances for reaction. No. 1 = 11/2 mm; No. 2 = 9/3 mm; No. 3 = 7/5 mm; N.o 4 = 5/7 mm; No. 5 = 3/9 mm; No. 6 = 2/11 mm. Based on this experiment, a distance of 4 mm was chosen for all subsequent double-diffusion and absorption studies.—Fig. 211. Experiment to determine the best cup-to-cup distances for reaction and to compare with cup-to-trough reactions. No. 1 = 15 mm; No. 2 = 2/11 mm. No. 3 = 3/9 mm; No. 4 = 5/7 mm; No. 5 = 7/5 mm. No. 6 = 9/3 mm; No. 7 = 11/2 mm; and No. 8 = 15 mm. Note that cup-to-trough gave better reactions even though the antigens were exactly the same concentration (8.15 mg protein/ml for C. sp.). In both Figures 210 and 211, antiserum C. sp. was used and both plates were photographed at 24 hr development.—Fig. 212. Experiment to determine the position effects of reactions in proximity with adjacent reactions, and position of cups and troughs on the agar plate. Antigen used: C. sp.—Fig. 213. Testing various concentrations and extraction procedures of *Chlorococcum* sp. (tetra) against antiserum C. sp. (E)

No. 1 = Whole powder, at protein conc. of 32.6 mg/ml
No. 2 = " " " " " " 16.3 "
No. 3 = " " " " " " 8.15 "
No. 4 = " " " " " " 4.7 "
No. 5 = " " " " " " 2.35 "
No. 5 = " " " " " " 2.35 "
No. 6 = 1st extract supernatant (10 min) from whole powder at protein conc. of 8.15 mg/ml
No. 7 = 2nd " " " " " " " " " " " " "
No. 8 = 3rd " " (overnight) " " " " " " " " " "
No. 9 = Plug of whole cells from which the 3rd extract was taken.

Plate photographed at 24 hr development.

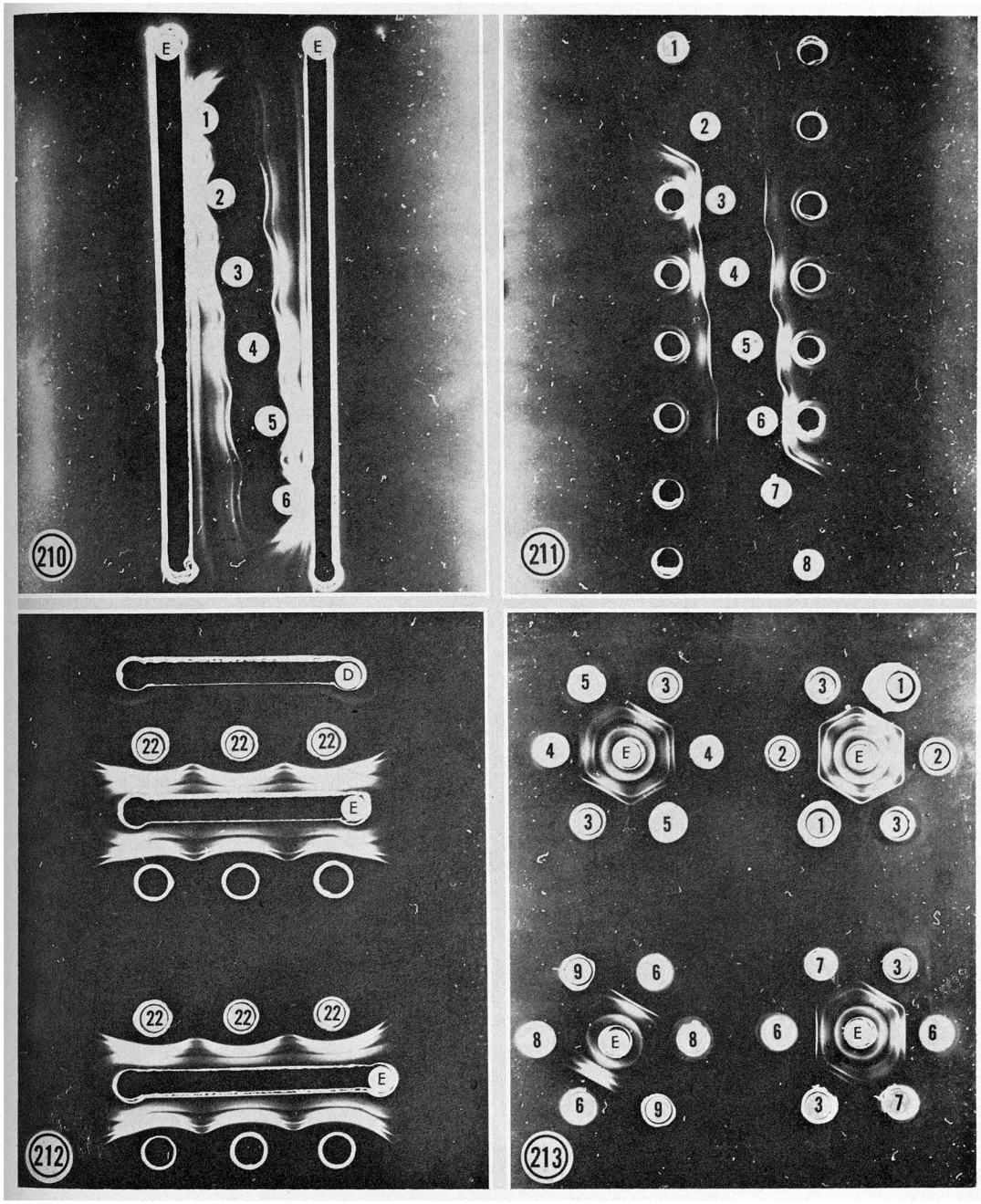
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Fig. 214–216. Double-diffusion reactions of all *Tetracystis* species. Plate was photographed at 24 hr development.

Fig. 214. No. 1 = *T. aeria* (C–6)
No. 2 = *T. aeria* (Pa–3)
No. 3 = *T. dissociata*
No. 4 = *T. isobilateralis*
No. 5 = *T. aggregata*
No. 215. No. 7 = *T. aplanosporum*
No. 8 = *T. pampae*
No. 9 = *T. intermedium*
No. 6 = *T. illinoisensis*
No. 7 = *T. aplanosporum*
Fig. 216. No. 10 = *T. excentrica*
No. 11 = *T. texensis*
No. 12 = *T. pulchra*
No. 22 = C. sp. (tetra)
No. 13 = *T. tetrasporum*

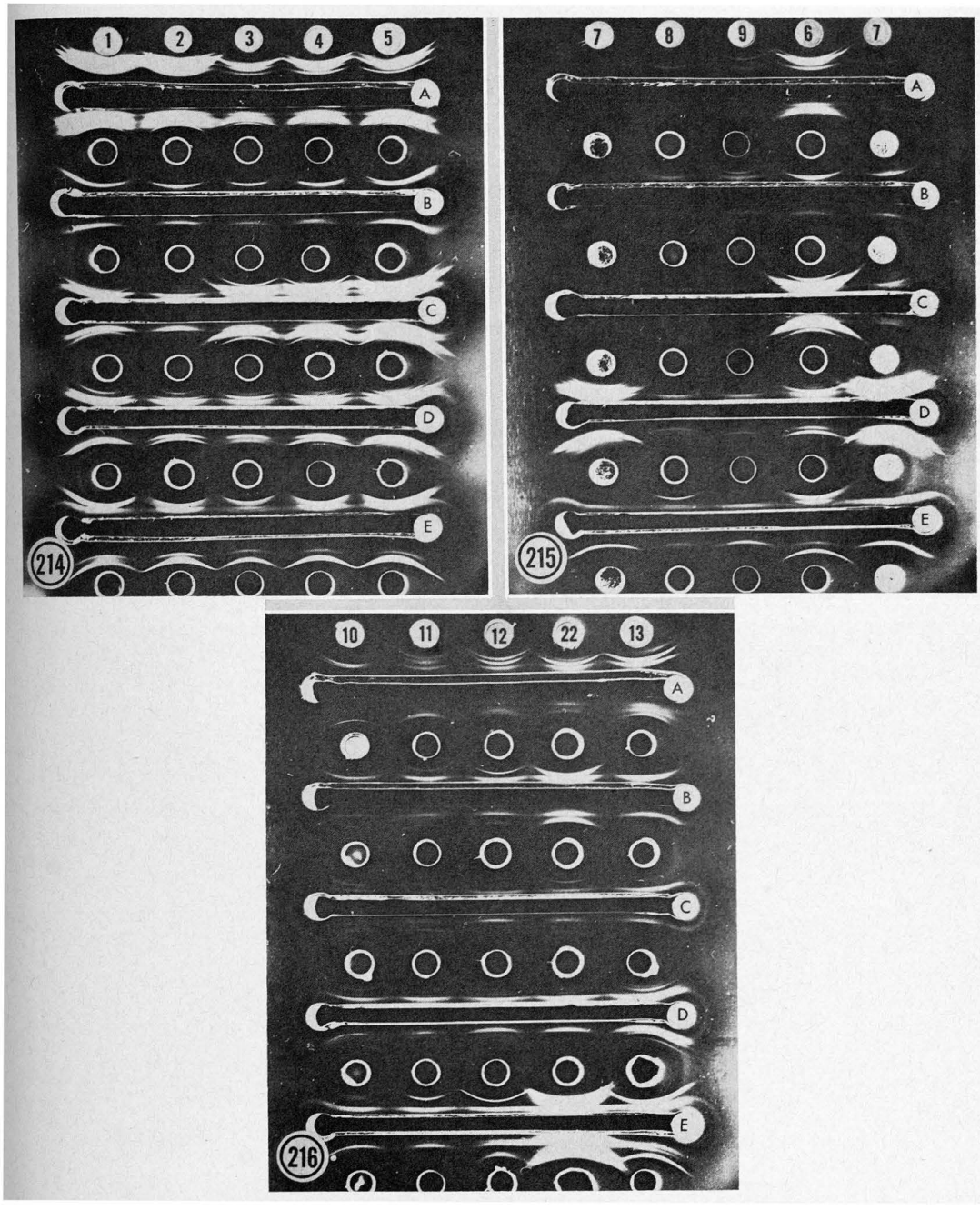
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Fig. 217–220. Double-diffusion reactions of *Chlorococcum* species. Plate was photographed at 24 hr development.

Fig. 217. No. 7 = *T. aplanosporum*
No. 15 = *C. diplobionticum*
No. 14 = *C. perforatum*
No. 16 = *C. echinozygotum*
No. 17 = *C. ellipsoideum*

Fig. 218. No. 18 = *C. hypnosporum*
No. 9 = *T. intermedium*
No. 14 = *C. perforatum*
No. 19 = *C. macrostigmatum*
No. 20 = *C. minutum*

Fig. 219. No. 21 = *C. multinucleatum*
No. 23 = *C. oleofaciens*
No. 14 = *C. perforatum*
No. 25 = *C. pinguidium*
No. 26 = *C. punctatum*

Fig. 220. No. 24 = *C. scabellum*
No. 22 = *C.* sp. (tetra)
No. 14 = *C. perforatum*
No. 27 = *C. vacuolatum*
No. 28 = *C. wimmeri*

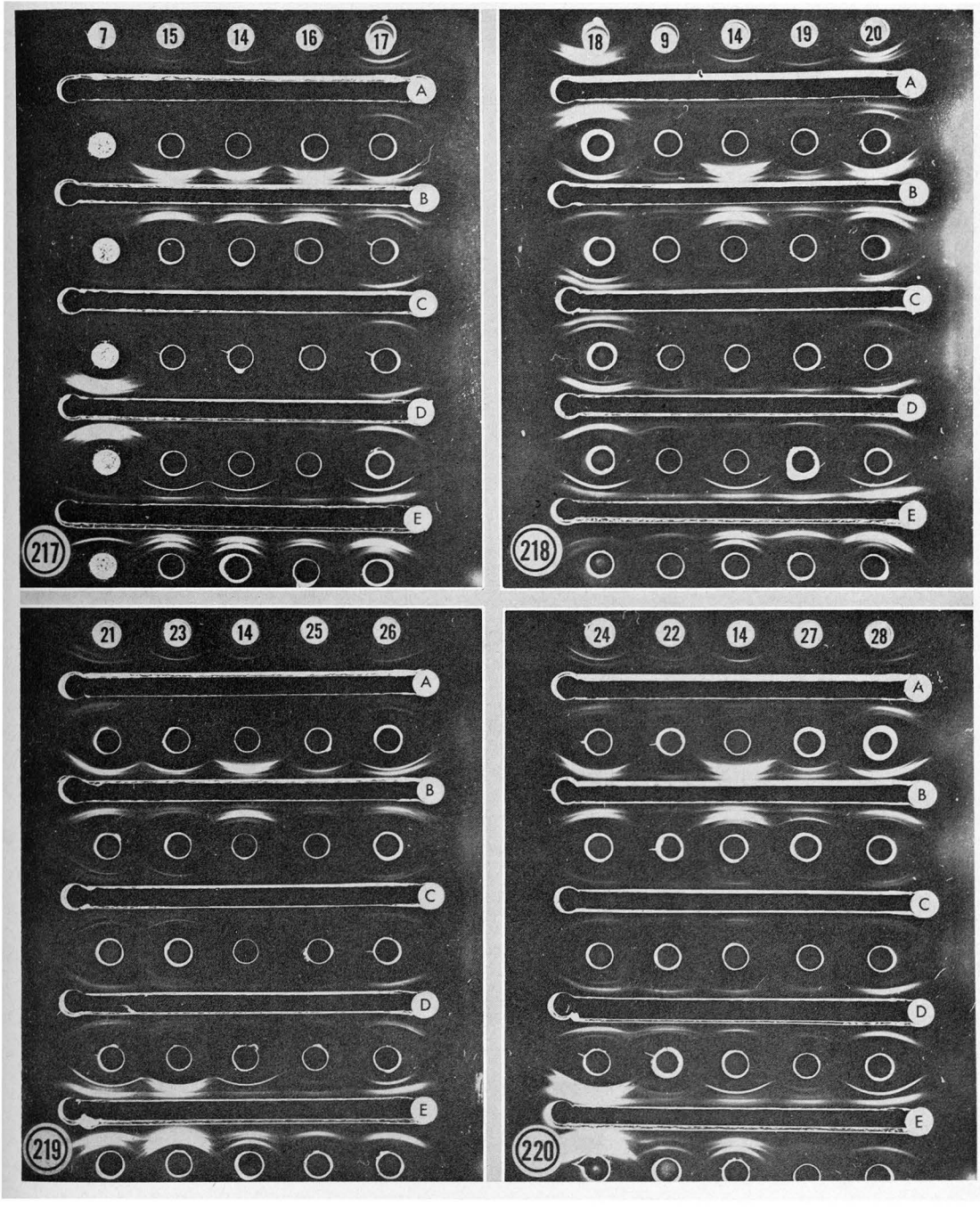
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Fig. 221–224. Double-diffusion reactions of *Chlorococcum* species with the same plates as in Fig. 222–225 but photographed at 48 hr development.

Fig. 221. No. 7 = *T. aplanosporum*
No. 15 = *C. diplobionticum*
No. 14 = *C. perforatum*
No. 16 = *C. echinozygotum*
No. 17 = *C. ellipsoideum*

Fig. 222. No. 18 = *C. hypnosporum*
No. 9 = *T. intermedium*
No. 14 = *C. perforatum*
No. 19 = *C. macrostigmatum*
No. 20 = *C. minutum*

Fig. 223. No. 21 = *C. multinucleatum*
No. 23 = *C. oleofaciens*
No. 14 = *C. perforatum*
No. 25 = *C. pinguidium*
No. 26 = *C. punctatum*

Fig. 224. No. 24 = *C. scabellum*
No. 22 = *C.* sp. (tetra)
No. 14 = *C. perforatum*
No. 27 = *C. vacuolatum*
No. 28 = *C. wimmeri*

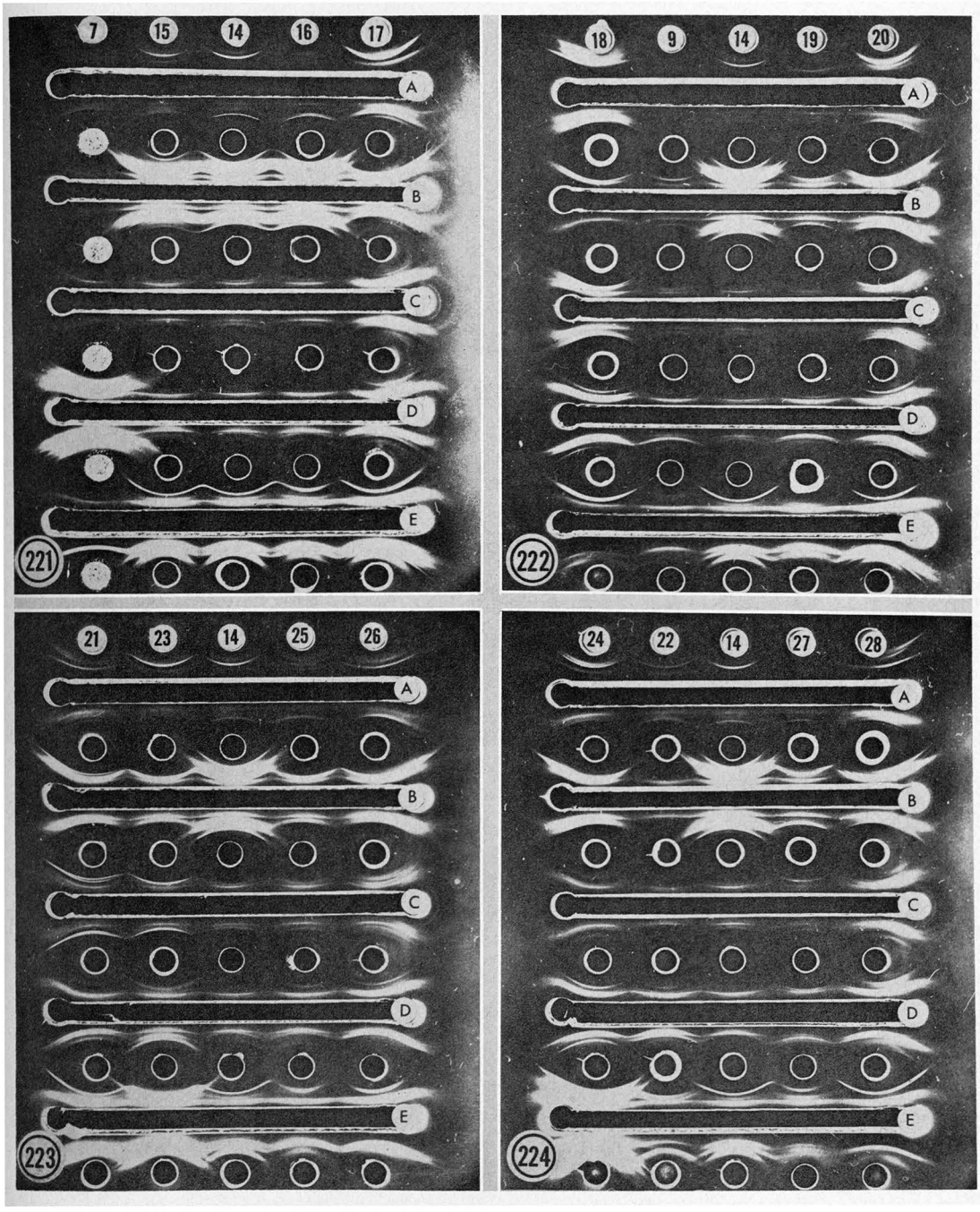
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Fig. 225–226. First-absorption study with the *T. aeria* and *T. isobilateralis* groups.

Fig. 225. Upper trough contains antiserum to *T. isobilateralis*, absorbed with *T. aeria* (Pa–3). Lower trough contains unabsorbed antiserum to *T. isobilateralis*.

No. 28 = *C. wimmeri*
No. 1 = *T. aeria* (C–6)
No. 3 = *T. dissociata*
No. 4 = *T. isobilateralis*
No. 2 = *T. aeria* (Pa–3)
No. 5 = *T. aggregata*
No. 6 = *T. illinoisensis*

Fig. 226. Upper trough contains antiserum to *T. aeria* (C–6) absorbed with *T. isobilateralis*. Lower trough contains unabsorbed antiserum to *T. aeria* (C–6).

No. 28 = *C. wimmeri*
No. 1 = *T. aeria* (C–6)
No. 3 = *T. dissociata*
No. 4 = *T. isobilateralis*
No. 2 = *T. aeria* (PA–3)
No. 5 = *T. aggregata*
No. 6 = *T. illinoisensis*

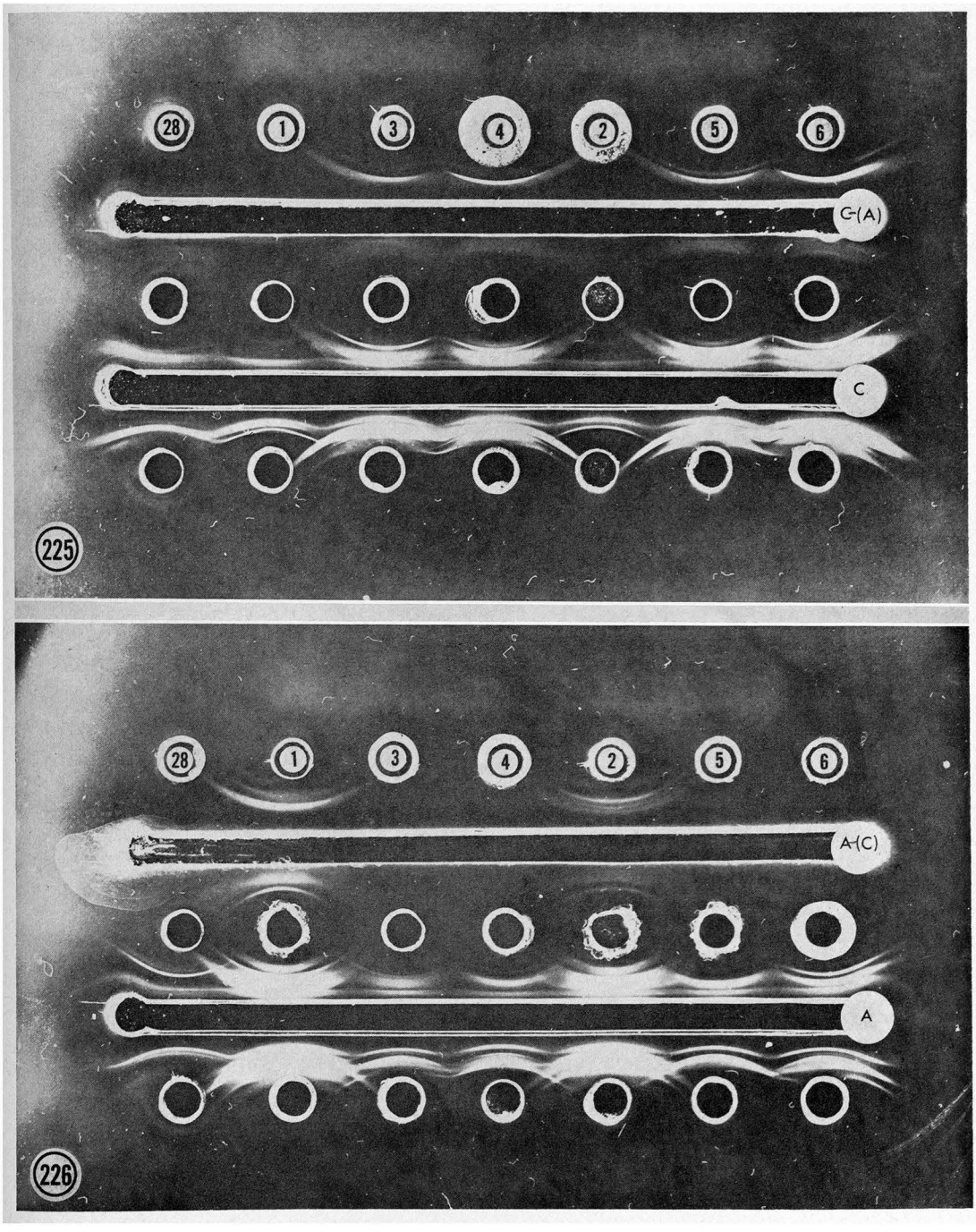
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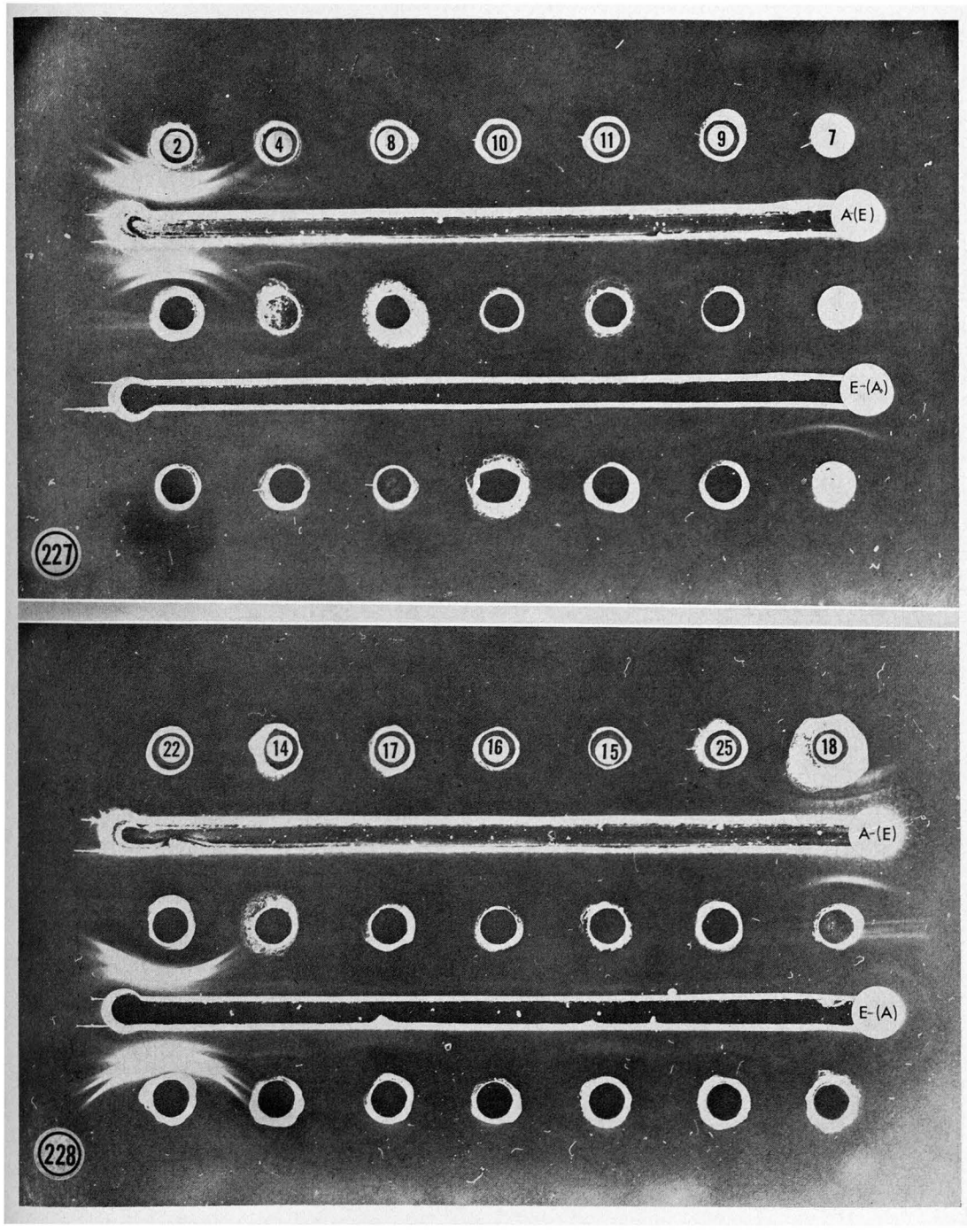
Fig. 227–228. Second-absorption study with selected species of *Chlorococcum and Tetracystis*.

Fig. 227. Upper trough contains antiserum to *T. aeria* (C–6) absorbed with C. sp. (tetra). Lower trough contains antiserum to C. sp. (tetra) absorbed with *T. aeria* (Pa–3).

No. 2 = *T. aeria* (PA–3)
No. 4 = *T. isobilateralis*
No. 8 = *T. pampae*
No. 10 = *T. excentrica*
No. 11 = *T. texensis*
No. 9 = *T. intermedium*
No. 7 = *T. aplanosporum*

Fig. 228. Upper trough contains antiserum to *T. aeria* (C–6) absorbed with C. sp. (tetra). Lower trough contains antiserum to C. sp. (tetra) absorbed with *T. aeria* (Pa–3).

No. 22 = C. sp. (tetra)
No. 14 = C. *perforatum*
No. 17 = C. *ellipsoideum*
No. 16 = C. *echinozygotum*
No. 15 = C. *diplobionticum*
No. 25 = C. *pinguidium*
No. 18 = C. *hypnosporum*


2
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11
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7
A-(E)
E-(A)
227
22
14
17
16
15
25
18
A-(E)
E-(A)
228

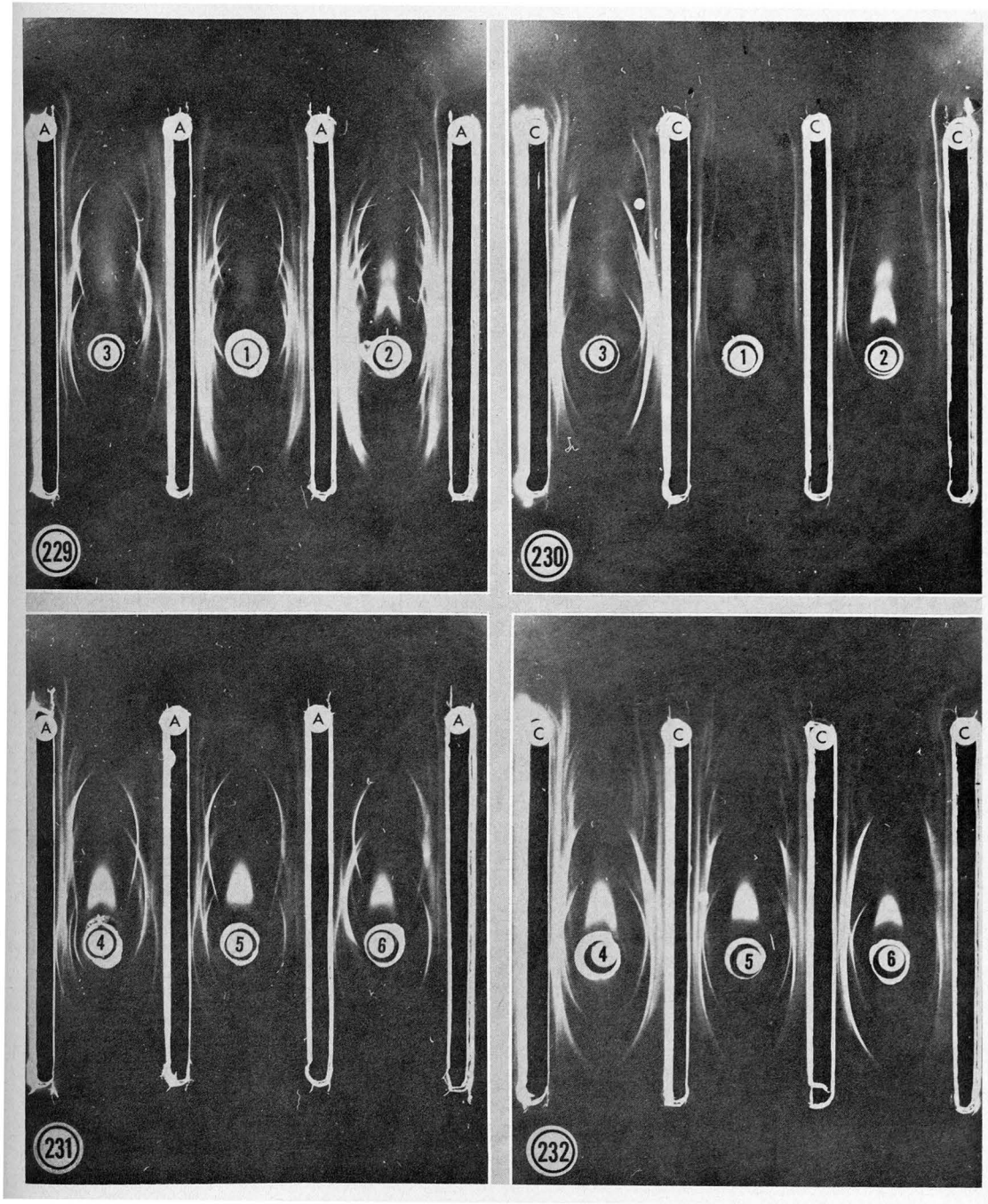
Fig. 229–232. Reactions of the *T. aeria* and *T. isobilateralis* groups in immunoelectrophoresis (photographed 24 hr in development).

Fig. 229. with antiserum to *T. aeria* (C-6). (A)
Antigens: No. 3 = *T. dissociata*
No. 1 = *T. aeria* (C–6)
No. 2 = *T. aeria* (Pa–3)

Fig. 230. With antiserum to *T. isobilateralis*. (C)
Antigens: No. 3 = *T. dissociata*
No. 1 = *T. aeria* (C–6)
No. 2 = *T. aeria* (Pa–3)

Fig. 231. With antiserum to *T. aeria* (C–6). (A)
Antigens: No. 4 = *T. isobilateralis*
No. 5 = *T. aggregata*
No. 6 = *T. illinoisensis*

Fig. 232. With antiserum to *T. isobilateralis*. (C)
Antigens: No. 4 = *T. isobilateralis*
No. 5 = *T. aggregata*
No. 6 = *T. illinoisensis*


A
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A
3
1
2
229
C
C
C
C
3
1
2
230
A
A
A
A
4
5
6
231
C
C
C
C
4
5
6
232

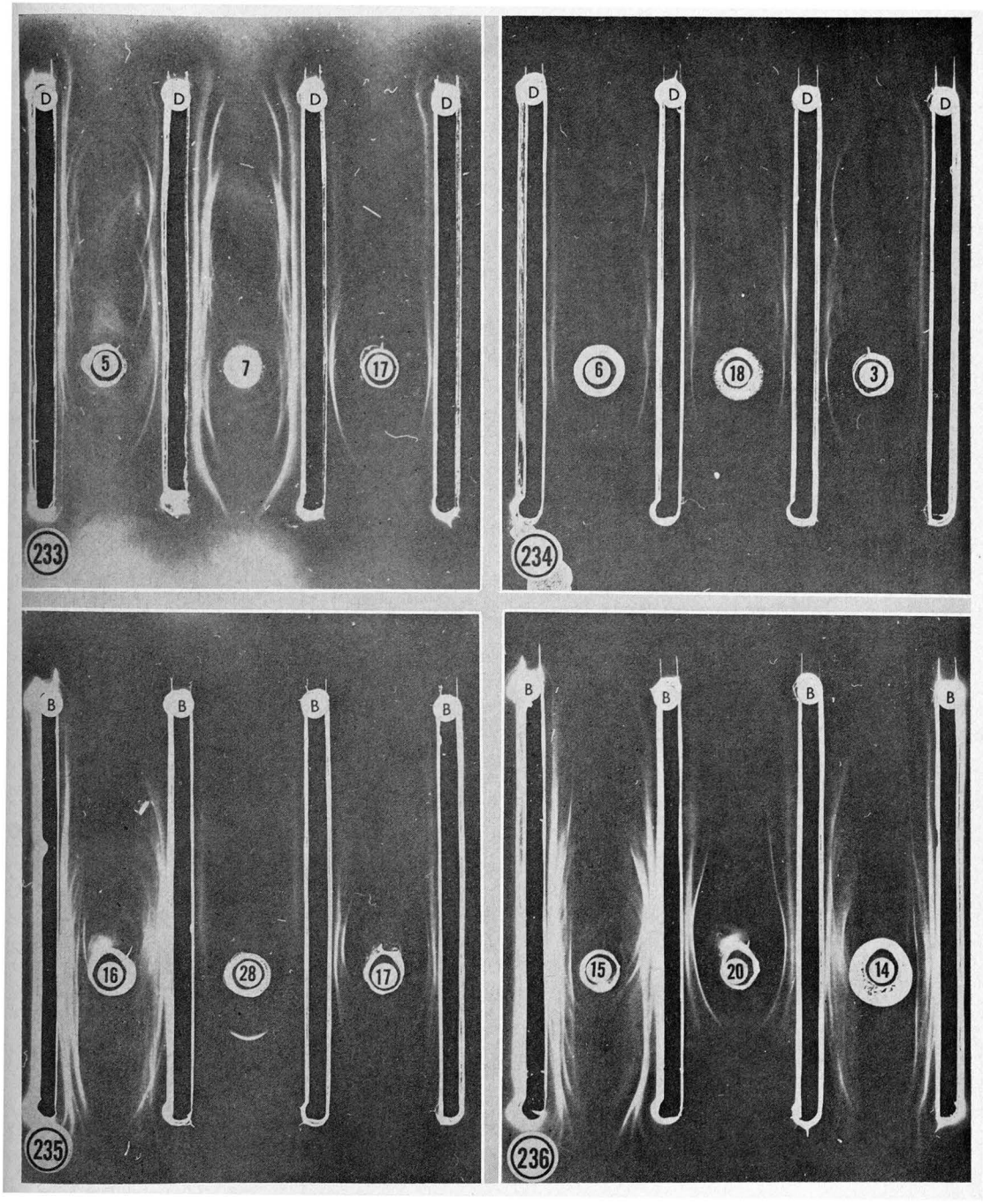
Fig. 233–236. Reactions of selected species of *Chlorococcum* and *Tetracystis* in immunoelectrophoresis (photographed 24 hr in development).

Fig. 233. With antiserum to *T. aplanosporum*. (D)
Antigens: No. 5 = *T. aggregata*
No. 7 = *T. aplanosporum*
No. 17 = *C. ellipsoideum*

Fig. 234. With antiserum to *T. aplanosporum*. (D)
Antigens: No. 6 = *T. illinoisensis*
No. 18 = *C. hypnosporum*
No. 3 = *T. dissociata*

Fig. 235. With antiserum to *C. perforatum*. (B)
Antigens: No. 16 = *C. echinozygotum*
No. 28 = *C. wimmeri*
No. 17 = *C. ellipsoideum*

Fig. 236. With antiserum to *C. perforatum*. (B)
Antigens: No. 15 = *C. diplobionticum*
No. 20 = *C. minutum*
No. 14 = *C. perforatum*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235
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B
B
15
20
14
236

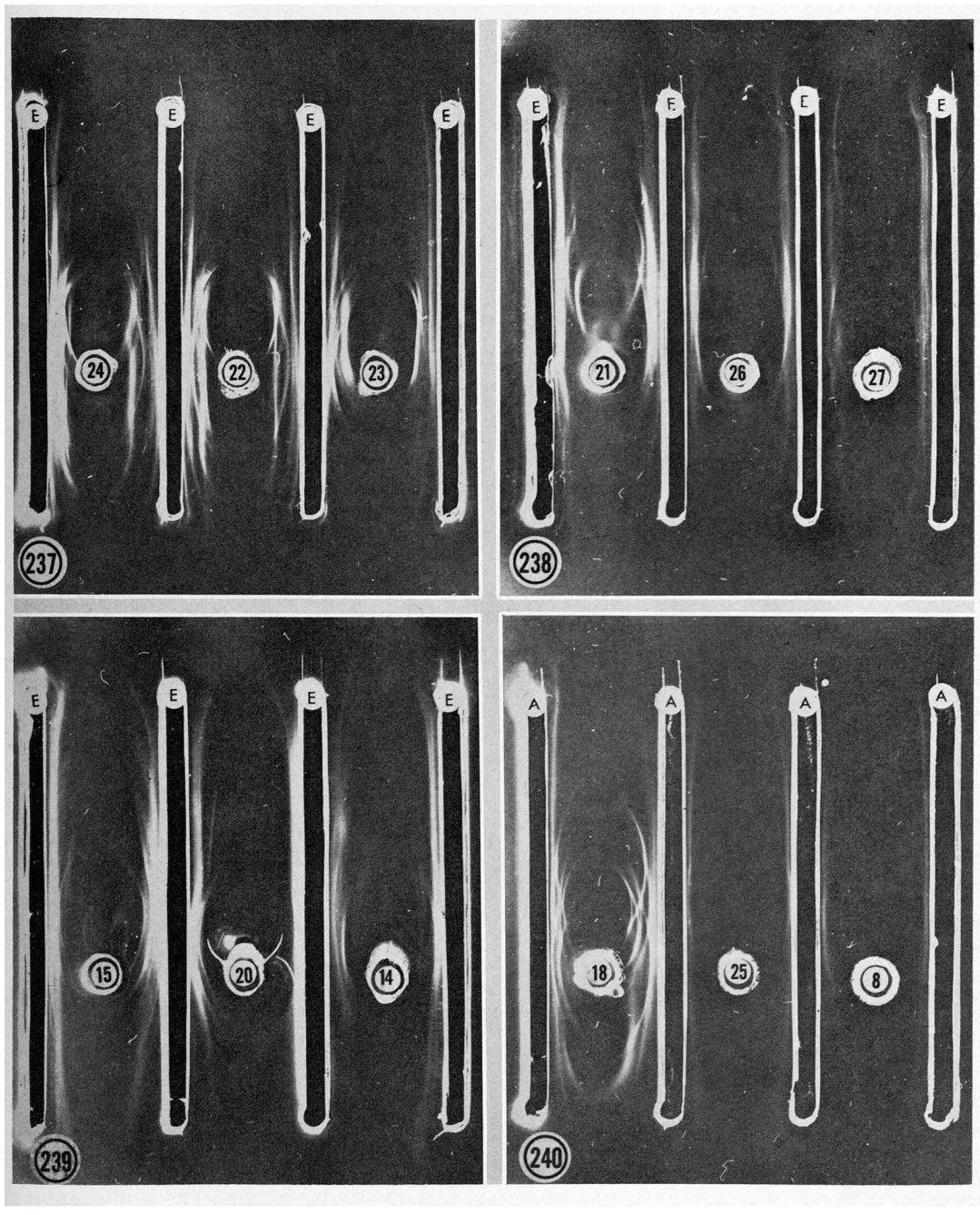
Fig. 237–240. Reactions of selected species of *Chlorococcum* and *Tetracystis* in immunoelectrophoresis (photographed 24 hr in development).

Fig. 237. With antiserum to C. sp. (tetra). (E)
Antigens: No. 24 = C. *scabellum*
No. 22 = C. sp. (tetra)
No. 23 = C. *oleofaciens*

Fig. 238. With antiserum to C. sp. (tetra). (E)
Antigens: No. 21 = C. *multinucleatum*
No. 26 = C. *punctatum*
No. 27 = C. *vacuolatum*

Fig. 239. With antiserum to C. sp. (tetra). (E)
Antigens: No. 15 = C. *diplobionticum*
No. 20 = C. *minutum*
No. 14 = C. *perforatum*

Fig. 240. With antiserum to *T. aeria* (C–6). (A)
Antigens: No. 18 = C. *hypnosporum*
No. 25 = C. *pinguidium*
No. 8 = *T. pampae*


E
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240